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SUBJECT OF INVESTIGATION

BIOCHEMICAL AND GENETICAL STUDIES

ON INH METABOLISM

RESPONSIBLE INVESTIGATOR

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Office of the Chief of Research and Development
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AD Div 16/11

Tokyo National Chest Hospital, Japan
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The capacity of acetylating in liver decreases in the order of chicken, rabbit, guinea pig and rat and after the addition of acetate to the reaction system a marked increase is observed

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3. Japan

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Japan
Taiwan
Tuberculosis
Blood plasma
Posage
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BIOCHEMICAL AND GENETICAL STUDIES ON ISONIAZID METABOLISM

Abstract

Trimodality of the frequency distribution curve of the biologically active isoniazid plasma level is observed not only in Japanese but also in Chinese in Formosa, Thai people and children between mixed Japanese and Caucasian or negro parentage, and cline in the frequency of the alleles controlling isoniazid inactivation from north to south is established with respect to various races in the Far East. Thus the validity of our genetical hypothesis on isoniazid inactivation has been verified again.

Correlation between isoniazid plasma level and clinical response during several kinds of isoniazid regimens is investigated. Although in these trials, 4 and 6 hour levels and area of time concentration curve after the fixed dose of 4 mg/kg body weight of isoniazid and a clinically prescribed single dose are taken in consideration, we are not successful in establishing a statistically significant difference in clinical efficacy between high and low dosage or high and low plasma concentration of isoniazid.

The capacity of acetylating isoniazid in liver homogenate decreases in the order of chicken, rabbit, guinea pig and rat and after the addition of acetate to the reaction system a marked increase is observed only in the former two kinds of animals. The site of the most active acetylation is the liver and our research into the intracellular localization of isoniazid acetylating enzyme reveals that it is contained in the main in the supernatant fluid. Only slight inactivation is observed in chicken liver homogenate with respect to PABA and sulfisoxazole and PABA is acetylated markedly by pigeon liver, while sulfisoxazole remained almost unchanged also in pigeon liver. But sulfanilamide is inactivated to a remarkable extent both by pigeon and chicken liver homogenate. Substrates which are located outside of TCA cycle like acetate, pyruvate, glucose and fructose accelerate acetylation of isoniazid by chicken liver homogenate. Addition of Co enzyme A enhanced acetylation in rat liver only to a limited extent and Co enzyme A contents of the livers of various kinds of animals are almost equal, regardless of varied degrees of acetylating capacity.

BIOCHEMICAL AND GENETICAL STUDIES ON ISONIAZID METABOLISM

Final Report No. 3

31 January 1963

Shigeichi Sunahara

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I. INTRODUCTION

Our Annual Reports of 1960 and 1961 demonstrated:

1. The vertical diffusion method developed in our laboratory is a quite reliable technique for determining the biologically active isoniazid concentration.

2. The value measured by this method varies from person to person but is constant for an individual.

3. Correlation of the blood levels between monozygous twins is so high that inactivation of isoniazid is beyond question a genetical trait.

4. The frequency distribution curve of 6 hour levels after ingestion of 4 mg/kg body weight of isoniazid in Japanese is trimodal contrary to the reports by American and European investigators who observed bimodal curves.

5. On the basis of natural distribution of the blood levels 6 hours after the dose of 4 mg/kg body weight of isoniazid, the following standard for classification of patterns of isoniazid inactivation is established.

Rapid inactivator	Less than or equal to 0.15 mcg/ml
Intermediate inactivator	Greater than 0.15 mcg/ml and less than 0.8 mcg/ml
Slow inactivator	Greater than or equal to 0.8 mcg/ml

6. Our pedigree study leads us to the following genetical hypothesis:

Inactivation of isoniazid is a charactor which is inherited without dominance, and rapid and slow inactivators are homozygous, while intermediate inactivators are heterozygous.

7. Population genetical study on not only Japanese but also various races in the Far East verified the above mentioned hypothesis.

8. The cline that the frequency of "slow" alleles increases from north to south is observed with regard to both different districts in Japan and various Asiatic countries.

9. Amount of free isoniazid excreted in urine runs parallel to a certain extent with the biologically active blood level, but the precise classification of patterns of isoniazid inactivation is impossible based on urinalysis.

10. Some correlation is established between inactivation of isoniazid and sulfonamide based on blood and urinalysis, but it is not the case with PABA and PAS.

11. After an increased dose of isoniazid, a higher blood level is obtained especially in rapid inactivators, but in the case of Japanese like in Eskimos, the increase is not so remarkable as in Caucasians.

12. Simultaneous administration of isoniazid with PAS or sulfonamide to the subject often raises the biologically active isoniazid concentration in blood plasma.

13. The amount of not only free isoniazid but also hydrazone excreted in urine of slow inactivator is greater than in intermediate and especially in rapid inactivator, and when test dose is raised the percentage of acetyl-isoniazid in urine decreases and in its place excretion of hydrazone increases.

14. Ingestion of isoniazid causes increased excretion of α-ketoglutaric acid and pyruvic acid in urine and the change is most remarkable in "slow" character. After the administration of both isoniazid and fructose, further increase of ketonic acid in urine can not be observed.

15. Blood level of the biologically active isoniazid after the administration of isoniazid derivatives remains longer than after the dose of isoniazid itself.

In the present Annual Report I am going to publish the results of our experiments during 1962 which deal with the following three problems concerning isoniazid inactivation:

1. Further population genetical study on some races in the Far East for the purpose of verifying our genetical hypothesis and at the same time our hypothesis on the geographical cline with respect to the frequency of the alleles controlling isoniazid acetylation.

2. Clinical studies to settle the much disputed problem as to whether there is a correlation between therapeutic effect and blood concentration of isoniazid.

3. Comparative studies on acetylation among various kinds of drugs, animal species and organs. Studies on intracellular localization of acetylating enzymes and influence of Co-A or several kinds of substrates on acetylation, etc.

II. LABORATORY METHOD

1. Vertical diffusion method for determining the biologically active isoniazid as described in the Annual Report 1961.

2. Hughe's modification of Short's method for measuring metabolites of INH as described in the Annual Report 1961.

3. Bratton-Marshall's method for measuring sulfonamide and PABA as described in the Annual Report 1961.

4. Schneider's method (1) for isolation of mitochondoria.

5. Warburg's method for studying the activity of acetylation and oxidation of tissue homogenate.

In the presence of the sufficient amount of ATP, Mg^{++} and phosphate, INH or other kinds of drugs is incubated with homogenate of liver or other tissue at 37° C. for 30 minutes.

Experimental condition for INH acetylation study in vitro:

Content of Warburg vessel		
Main chamber	1/15 M phosphate buffer	0.3 ml
	0.01 M Na-A.T.P.	0.3
	0.1 M MgCl	0.1
	0.4 M NaF	0.1
	0.1 M Na-Acetate	0.4
	500 γ/ml INH	0.4
	0.4 g homogenate	1.0
	H_2O	0.4
	Total	3.0
Side chamber	20% KOH	0.2

Temperature: 37° C. Gas phase: Air Incubation: 30 minutes

6. Kaplan-Lipmann's method (2) for determining Co enzyme A

a. Preparation of aged liver extract

Minced pigeon livers are transfered to a chilled Waring mixer with a volume of chilled acetone for two minutes. The homogenate is rapidly filtered on a Büchner funnel and the residue is washed on the funnel with acetone and ether. The powder is dried in a vacuum desiccator over phosphate pentoxide. Ten g of liver powder is rubbed up with 100 ml of 0.02 M Nabicarbonate solution, and kept overnight in a deep freezer after centrifugation. After aging in room temperature for 4 hours, the extract is centrifuged and the supernatant is used as crude enzyme solution. Both transacetylase and acetokinase are contained in the extract.

b. Preparation of standard Co enzyme A

Fresh minced rabbit liver is boiled with 3 volumes of water, brought to Ph2 with hydrochloric acid and then precipitated with 10 volumes of acetone. The precipitate is washed with acetone and dried with ether.

c. Assay method

(1) Reaction mixture

0.004 M	Sulfanilamide	10.0 ml
1.0 M	Na acetate	2.5 ml
0.05 M	K-ATP	8.0 ml
0.5 M	Na-citrate	10.0 ml

(2) Reaction assay

Solution to be tested for Co enzyme A	0.3 ml
Reaction mixture mentioned above	0.3 ml
1 M $NaHCo_3$ (Co_2 standard)	0.08 ml
0.1 M cystein hydrochloride	0.1 ml
Aged enzyme solution	0.25 ml

The tube is stopped, mixed and incubated for 2 hours in a water bath of 37° C. After the reaction is halted by adding 4 ml of 5% trichloroacetic acid, the amount of sulfanilamide is measured by Bratton-Marshall's method.

(3) Standard curve

Concentration-activity curves for Co enzyme A is prepared with standard Co enzyme A preparation as described later (see P. 58).

III. EXPERIMENTAL RESULTS

1. POPULATION GENETICAL AND ANTHROPOLOGICAL STUDY

a. Trimodality of frequency distribution curve of biologically active isoniazid levels of children between mixed Japanese and Caucasian or negro parentage

As indicated in Table 1, 63 half-bloods were examined and the result is compiled in Table 2. It was worthy of notice that "Intermediate" offspring were remarkably frequent in comparison with the mating between Japanese, and the trimodal distribution was established also in the case of the half-bloods as shown in Fig. 1.

b. Trimodality of frequency distribution curve of biologically active isoniazid levels of Chinese and Thai people

Although the trimodality of frequency distribution curve of the microbially active isoniazid plasma levels was repeatedly observed by us as far as Japanese were concerned, investigators who examined Caucasians or negroes seemingly failed to observe a trimodal curve. Therefore, it was necessary for us to determine the blood level of isoniazid in Caucasians by means of our own laboratory method but as we were not successful in making such a study so far, we intended in the present report to take blood samples of Asiatic people such as Chinese and Thai which showed higher frequency of "Slow" characters, and were more close, in this respect, to the Whites than in the case of Japanese. As to Thai people, we published already in the former report the result of our previous research, and it is interesting to notice that the two values for frequency of "Slow" alleles measured by us on two different occasions (1961 and 1962) coincided with each other fairly well: 0.5417 in the investigation of 1961 and 0.5408 in the present study. The reproductibility is so well that we may reasonably conclude that our laboratory technique for measuring the biologically active isoniazid is highly reliable.

Fig. 2 and 3 demonstrate that segregation of the blood levels into three groups as we pointed out in Japanese is also true with Chinese and Thai, and Fig. 4 and 5 illustrate that the frequency distribution curves are trimodal as in the case of Japanese.

c. Cline in frequency of alleles controlling isoniazid inactivation among several Asiatic races

The trend that the more southerly a country or a land is located, the higher is the frequency of "Slow" alleles in the order of Ainus, Koreans, Japanese Ryukuans, Chinese in Formosa and Thai people is revealed in Fig. 6. The almost same kinds of "cline" was reported by us already with respect to the inhabitants in various regions of Japan in the Annual Report of 1961 and elsewhere (Sunahara (3,4)).

Table 1 Biologically active isoniazid concentration of half-bloods (6 hour level after dose of 4 mg/kg)

Race of father	White				Negro				
				Rapid					
No. 1	Age 7	Sex	F	0.182 mcg/ml	No. 1	Age 9	Sex	M	0.174 mcg/ml
2	7		F	0.164	2	9		M	<0.1
3	9		M	0.121	3	9		M	<0.1
4	9		M	<0.1	4	9		M	<0.1
5	11		F	0.118	5	11		M	<0.1
6	13		M	<0.1	6	11		M	<0.1
7	14		M	0.132	7	11		F	<0.1
8	14		M	0.124	8	11		F	<0.1
					9	13		M	<0.1
					10	13		M	<0.1
					11	15		M	<0.124
				Intermediate					
No. 1	Age 9	Sex	M	0.22 mcg/ml	No. 1	Age 9	Sex	M	0.318 mcg/ml
2	13		M	0.299	2	10		M	0.51
3	13		F	0.51	3	10		M	0.21
4	13		M	0.2	4	11		M	0.4
5	14		F	0.318	5	11		M	0.265
6	14		F	0.23	6	11		F	0.728
7	14		M	0.7	7	11		M	0.48
8	14		M	0.21	8	11		M	0.2
9	14		M	0.54	9	11		M	0.31
10	14		M	0.54	10	11		M	0.29
11	14		M	0.66	11	11		M	0.20
12	14		M	0.57	12	12		M	0.70
13	15		M	0.34	13	12		M	0.20
					14	13		F	0.42
					15	13		F	0.21
					16	13		M	0.21
					17	13		M	0.21
					18	13		M	0.21
					19	14		F	0.318
					20	14		M	0.36
					21	14		F	0.45
					22	14		M	0.23
					23	14		M	0.21
					24	14		M	0.25
					25	15		M	0.34
					26	15		M	0.31
				Slow					
No. 1	Age 11	Sex	F	1.18 mcg/ml	No. 1	Age 11	Sex	F	1.24 mcg/ml
2	14		M	1.35	2	13		M	1.48
3	14		F	1.32					

Table 2 Frequency of three types of isoniazid inactivation among half-breeds

Mating	Rapid		Intermediate		Slow		Total	
	No.	%	No.	%	No.	%	No.	%
Japanese x Negro	1	28.5	26	66.5	2	5.0	39	100
Japanese x White	8	33.3	13	54.2	3	12.5	24	100
Japanese x Japanese	798	44.1	803	44.4	207	11.5	1808	100

Fig. 1 Frequency distribution curve of isoniazid blood level of half-bloods (6 hours after 4 mg/kg dose)

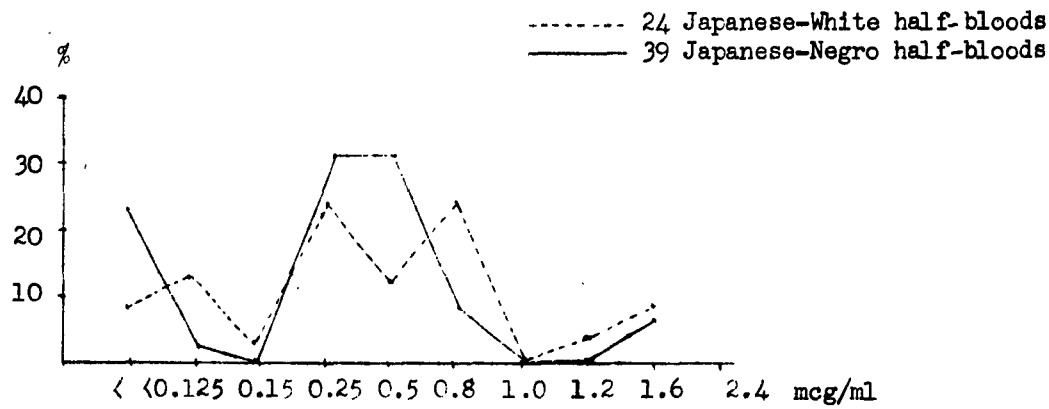


Fig. 2

Relationship between 6 and 8 hour blood levels after administration of 4 mg/kg of INH to Chinese in Formosa

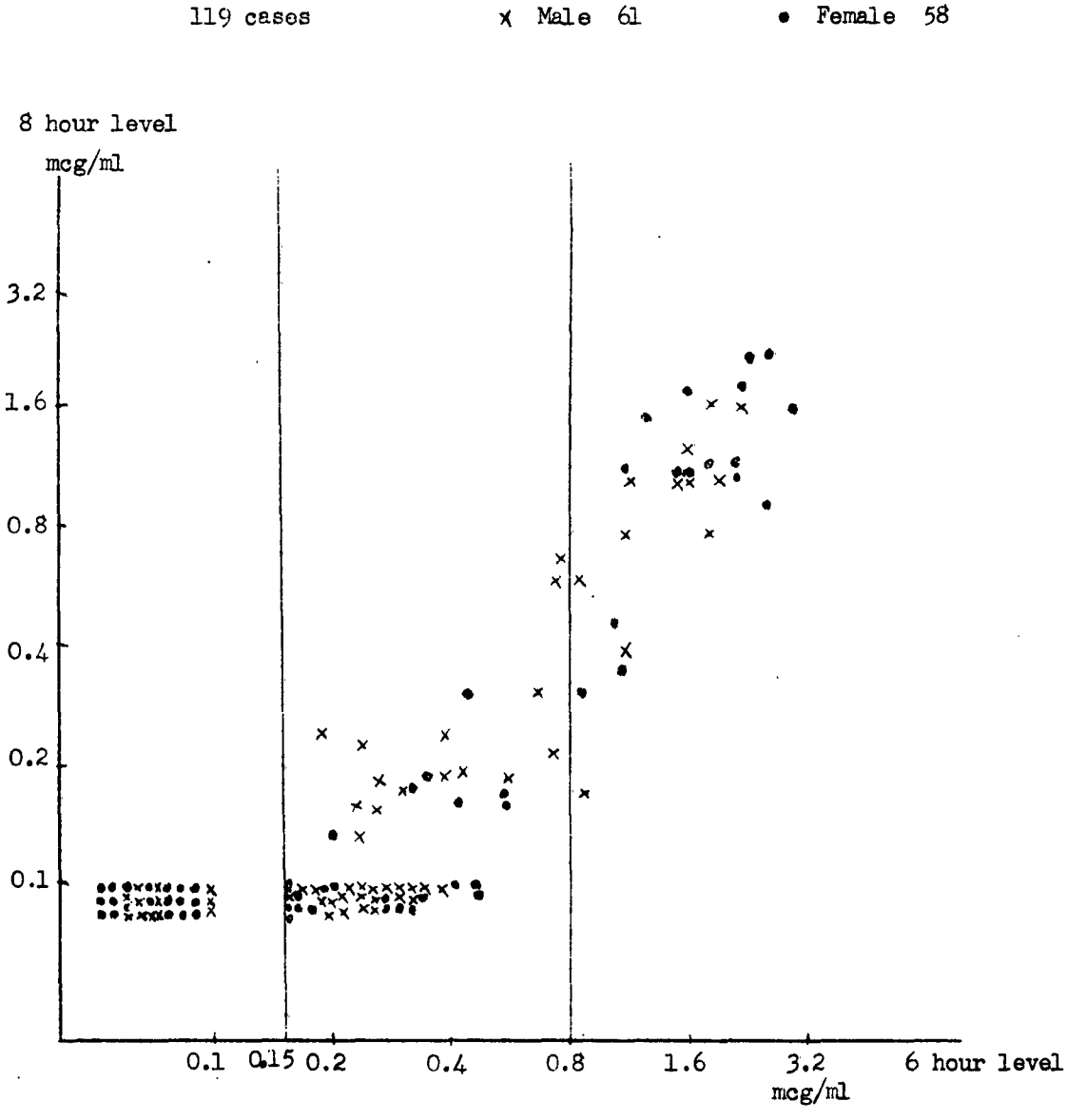


Fig. 3

Relationship between 6 and 8 hour blood levels after administration of 4 mg/kg INH to Thailanders

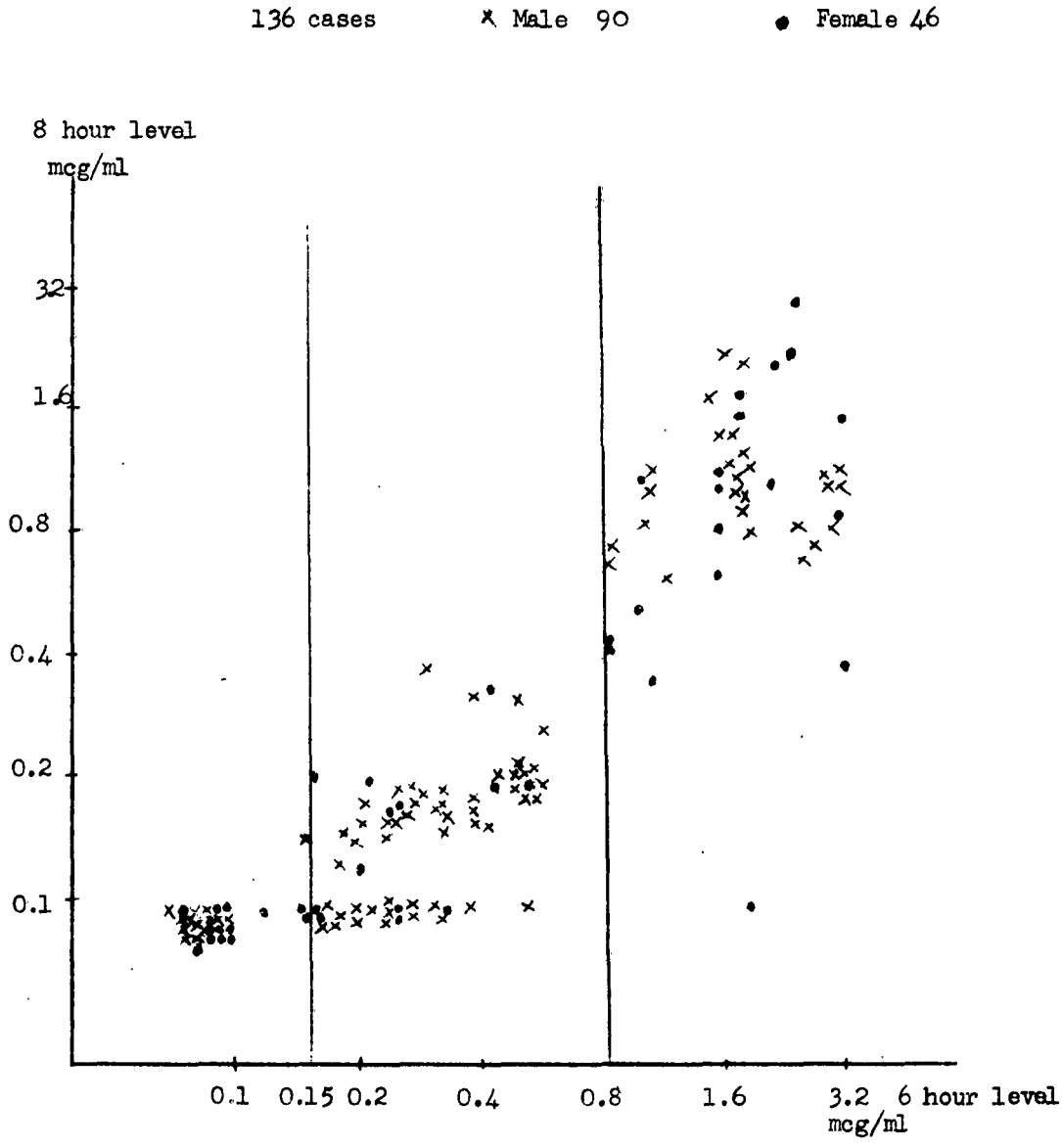


Fig. 5

Distribution curve of blood levels at 6 and 8 hours after administration of 4 mg/kg INH to Thailanders

Number of subjects	Male	Female	Unknown	Total
6 hr level	109	73	2	184
8 hr level	107	71	1	179

Classification	
R:	46 (25.0)
I:	77 (41.8)
S:	61 (33.2)
Total:	184 (100%)

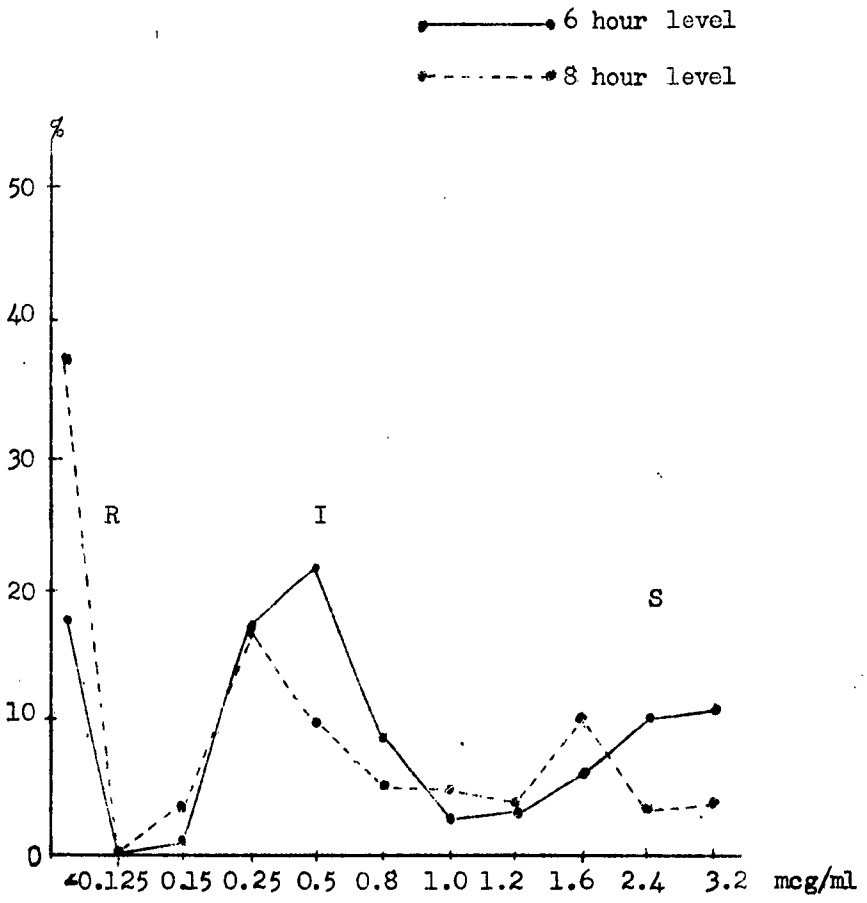
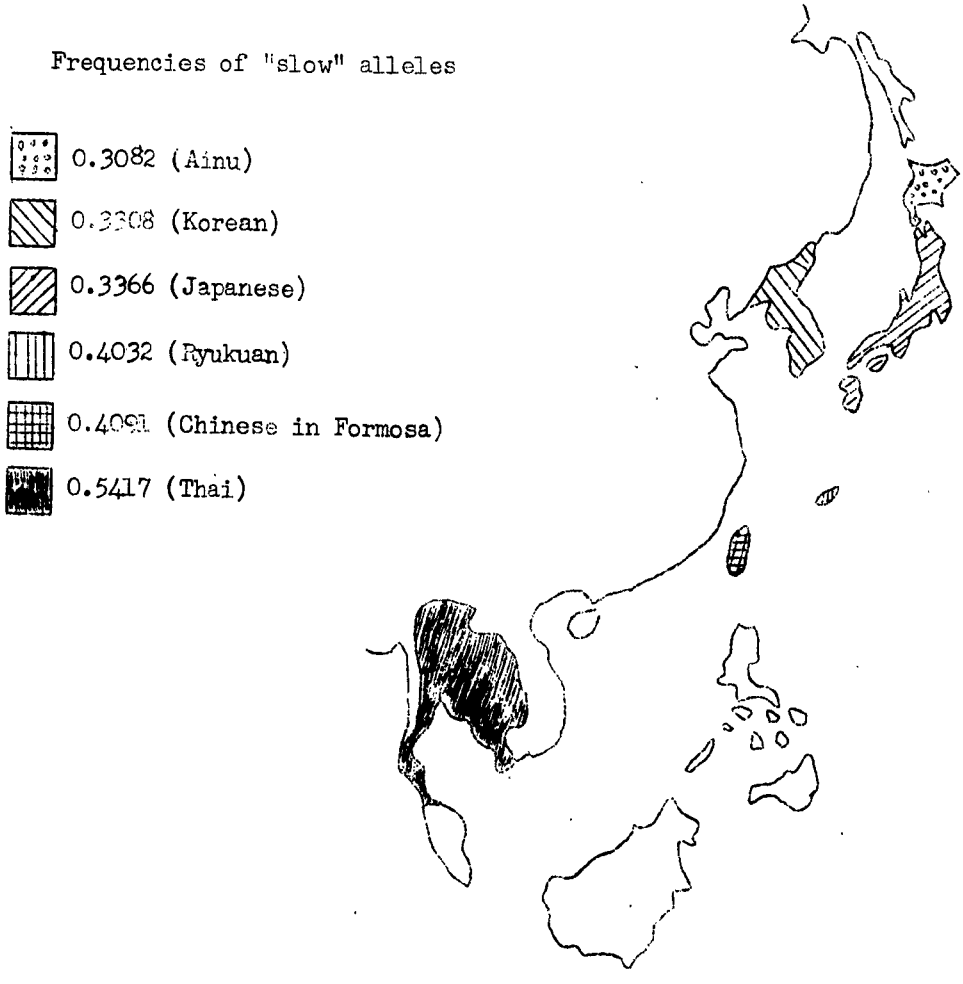


Fig. 6 Cline in frequency of alleles controlling isoniazid metabolism among several races in the Far East



2. CORRELATION BETWEEN ISONIAZID PLASMA LEVEL AND CLINICAL EFFECT OF ISONIAZID TREATMENT OF PULMONARY TUBERCULOSIS

The subjects in this investigation were a part of pulmonary tuberculosis patients who were admitted to the Controlled Trials of the National Sanatorium Cooperative Study. They were primary treatment cases and allocated at random to several kinds of isoniazid regimens.

Patients admitted to three independent series of trials were involved in this study. The first series consisted of the following three regimens and dealt with the fixed test dose of 4 mg/kg body weight of isoniazid.

1) INH 200-300 mg daily + PAS 10 g daily:
) 104 cases
2) INH 200-300 mg daily + PZA 3 g daily:

3) INH 200-300 mg daily + sulfisoxazole or sulfisomidine 2 g daily: 127 cases

The second series was concerned with the regimen of isoniazid 200-300 mg daily + PAS 10 g daily (126 cases) and the fixed dose of 4 mg/kg body weight of isoniazid was given to the patients of this series. The regimens in the third series consisted of:

1) INH 200-300 mg daily + PAS 10 g daily:

2) INH 18 mg/kg daily + PAS 10 g daily:

and the blood levels after the dose identical to a single dose which was administered clinically to the individual patient during treatment, were determined.

In the present research, the concentration of isoniazid in blood plasma was expressed in five different ways: 4 hour level, 6 hour level, mean of these two values, area of time concentration curve (sustained level) and calssification into three patterns of isoniazid inactivation (slow, intermediate and rapid inactivation).

As the clinical effect of INH+PZA and INH+PAS treatment in the first series turned out to be almost equal, we treated these two regimens together in Fig. 7-11. Fig. 12-14 dealt with INH+sulfa-drug treatment and Fig. 15-18 with all these three kinds of regimens.

In the first series, we were not successful in establishing a definite difference in therapeutic effect related to the plasma level of the biologically active isoniazid concentration. Also in the second series of the trial (Fig. 19-22), the higher levels after the fixed dose of 4 mg/kg body weight of isoniazid did not seem to have any advantage over the lower levels with regard to sputum conversion, but the rate of regression of cavity at the 3rd and 6th month were a little higher in the higher concentration group than in the lower concentration group. In the third series (Fig 23-27) in which a single therapeutic dose was administered as a test drug, some relationship between the rate of improvement of non-cavitary and especially cavitary lesion on the one hand and the blood levels on the other was revealed, But the difference related to the blood levels was not statistically significant.

There were a lot of reports concerned with comparison of therapeutic efficacy between ordinary and high dose of isoniazid, but it seemed very difficult to prove conclusively the advantage of high dose regimen over ordinary dose regimen. Therefore, for the purpose of comparing the clinical effect of the ordinary dose regimen with the very low dose regimen, we tried to allocate 51 pulmonary tuberculosis patients without previous chemotherapy to the following two groups.

a) 4 mg/kg of isoniazid in two divided doses

b) 2 mg/kg of isoniazid in two divided doses

As the average body weight of Japanese who were suffering from pulmonary tuberculosis was about 50 kg, a single clinical dose of "b" group was only 50 mg. Although a random allocation was made, the number of examined cases were so small that the percentage of rapid inactivators was by chance greater in the higher dose group. Consequently no appreciable difference was established between these two regimens as shown in Fig 28. Then we classified the subjects into two groups based on the isoniazid blood level, but again we failed to establish a significant difference between the cases with the blood level above zero and the cases with no measurable amount of isoniazid in blood.

Fig. 7

Relationship between clinical effect of INH-PAS & INH-PZA regimens and blood level 4 hours after 4 mg/kg dose of INH (98 Subjects)

Comparability of different groups

Blood level		≤0.5 γ	>0.5 γ
Total number		52 100%	46 100%
Sex	Male	67.3	74.0
	Female	32.7	26.0
Age	-20	21.2	15.2
	21-50	65.4	69.8
	51-	13.4	15.2
NTA Classification			
Min.		9.6	13.1
Mod. adv.		40.4	34.9
Far adv.		50.0	52.0
Noncavitary lesion			
Infiltrative caseous		81.0	84.7
Fibrocaseous		15.2	10.9
Far adv. mixed form		3.8	4.4
Cavity			
No cavity		15.3	10.8
Single cavity			
Sclerotic		7.7	4.3
Nonsclerotic		46.3	26.1
Multiple cavity			
Sclerotic		5.7	4.3
Nonsclerotic		25.0	54.5

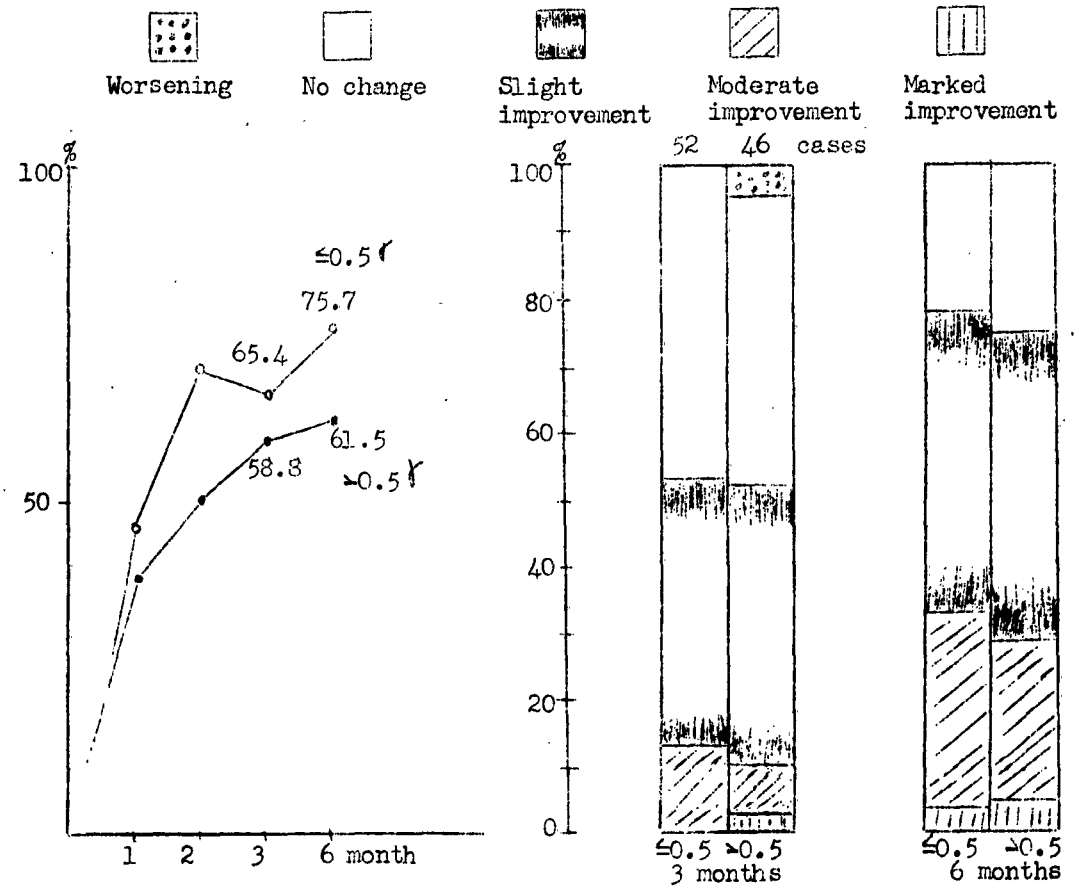
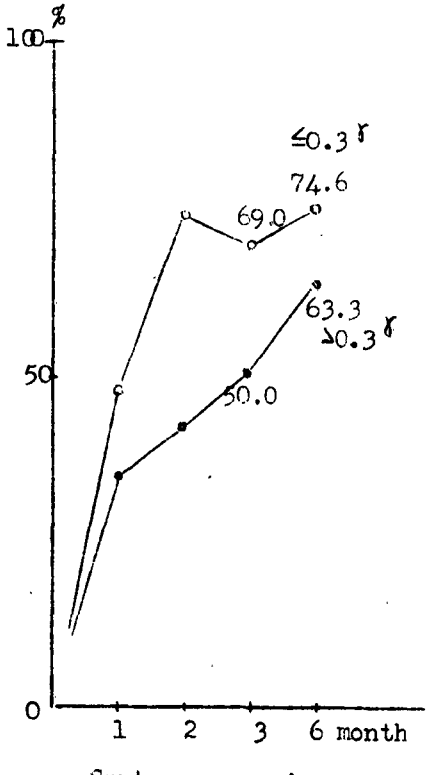


Fig 8

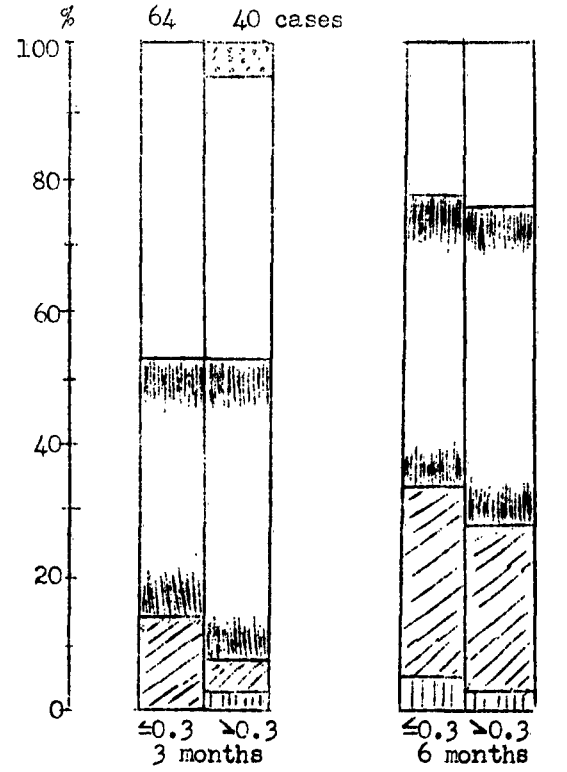
Relationship between clinical effect of INH treatment (INH-PAS & INH-PZA) and blood level 6 hours after dose of INH (104 subjects)

Comparability of different groups

Blood level	≤0.3 γ	>0.3 γ
Total number	64 100%	40 100%
Sex Male	68.7	77.5
Sex Female	31.3	22.5
Age -20	23.5	12.5
Age 21-50	62.2	77.0
Age 51-	14.3	10.5
NTA Classification		
Min.	9.4	12.5
Mod. adv.	42.2	32.5
Far adv.	48.4	55.0
Noncavitary lesion		
Infiltrative caseous	79.7	87.5
Fibrocaseous	18.8	7.5
Far adv. mixed form	1.5	5.0
Cavity		
No cavity	18.8	7.5
Single cavity		
Sclerotic	4.7	5.0
Nonsclerotic	40.6	27.5
Multiple cavity		
Sclerotic	6.2	15.0
Nonsclerotic	29.7	45.0



Sputum conversion



Improvement of noncavitary lesion

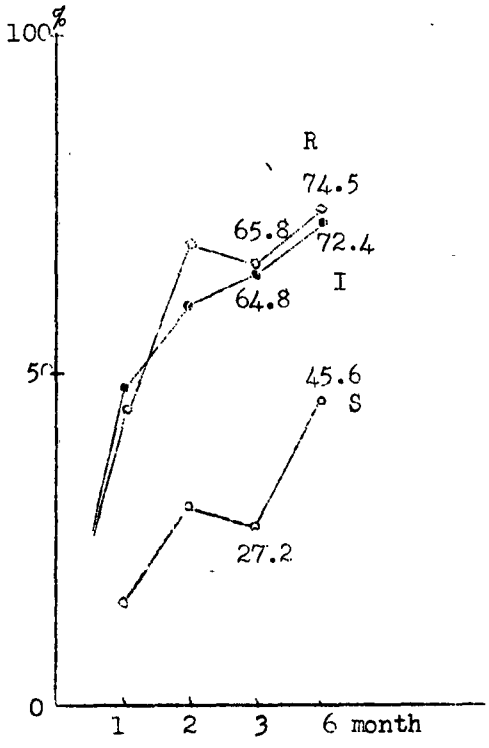
Fig. 9

Relationship between clinical effect of INH treatment (INH-PAS & INH-PZA) and pattern of INH inactivation (104 subjects)

Comparability of different groups

Pattern of INH inactivation		R	I	S
Total number		42 (100%	51 100%	11 100%)
Sex	Male	66.6	74.6	81.9
	Female	33.4	25.4	18.1
Age	-20	21.4	19.6	9.0
	21-50	62.0	70.6	64.0
	51-	16.6	9.8	27.0
NTA Classification				
Min.		7.2	13.7	9.0
Mod. adv.		45.3	41.2	0
Far adv.		47.5	45.1	91.0

	R	I	S
Noncavitary lesion			
Infiltrative caseous	77.1	82.5	91.0
Fibrocaseous	19.1	13.6	0
Far adv. mixed form	3.8	3.9	9.0
Cavity			
No cavity	14.3	15.8	9.0
Single cavity			
Sclerotic	7.1	9.8	0
Nonsclerotic	50.0	31.3	0
Multiple cavity			
Sclerotic	4.8	9.8	27.0
Nonsclerotic	23.8	33.3	64.0



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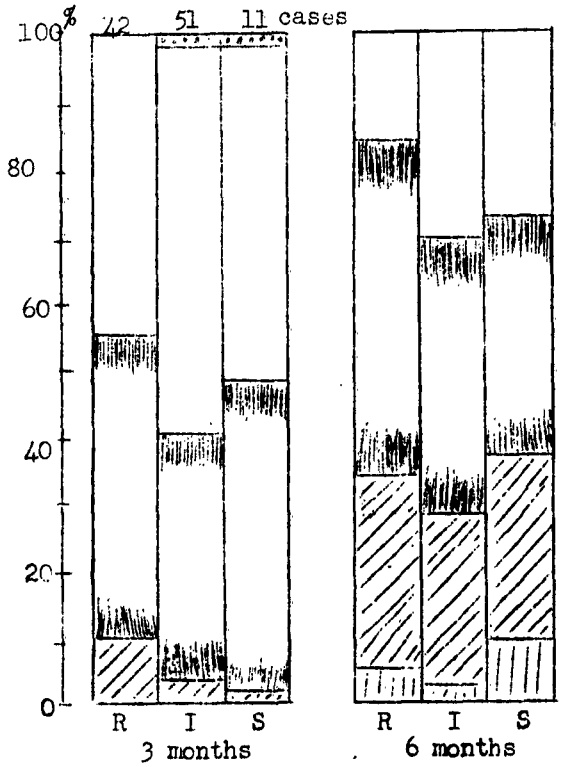


17 Improvement of noncavitary lesion

Fig. 10

Relationship between regression of nonsclerotic cavity in INH-PAS & INH-PZA treatment and blood level after 4 mg/kg dose of INH (100 subjects)

Comparability of different groups

Cavity	Pattern of inactivation R	I	S	6 hour level ≤0.3	>0.3	4 hour level ≤0.5	>0.5
Round	29 (76.3%)	26 (55.1)	4 (26.7)	38 (66.7)	21 (48.8)	29 (66.0)	28 (52.7)
Irregular	9 (23.7%)	21 (44.9)	11 (73.3)	19 (33.3)	22 (51.2)	15 (34.0)	25 (47.3)

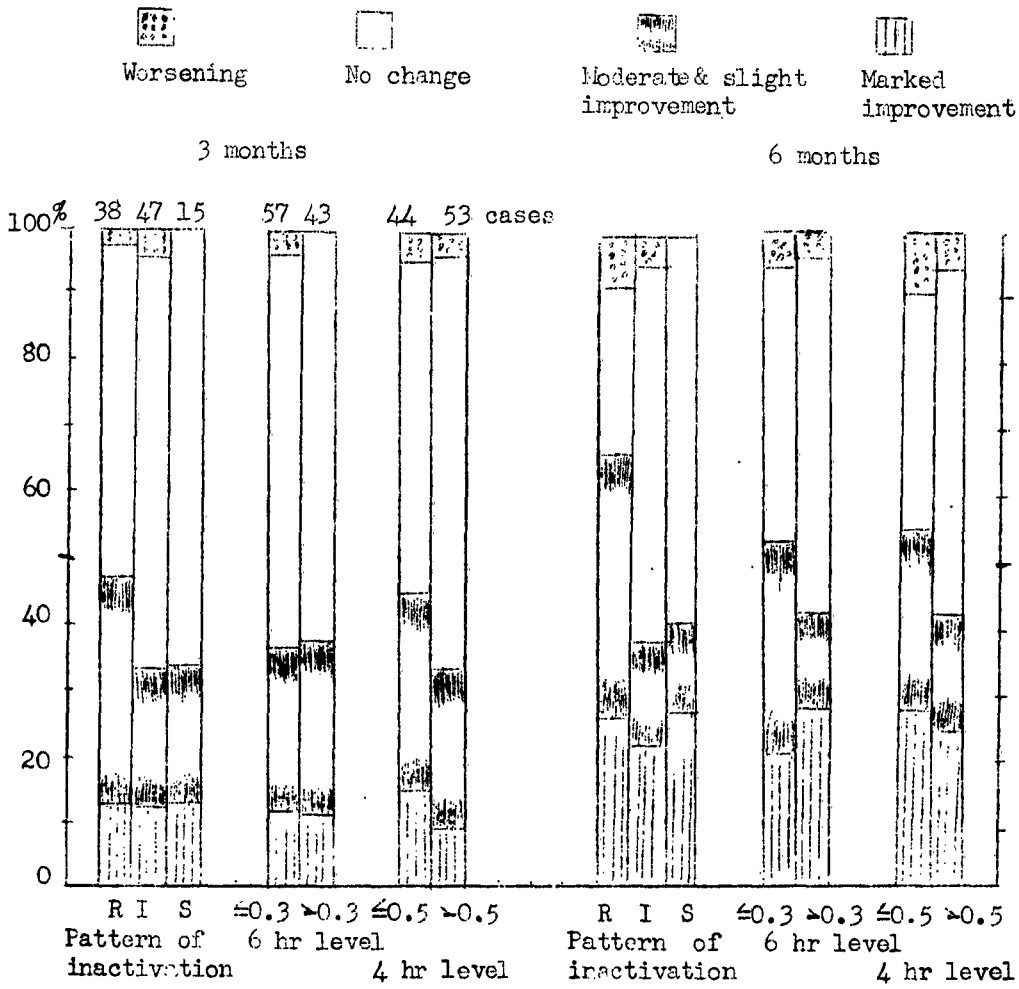


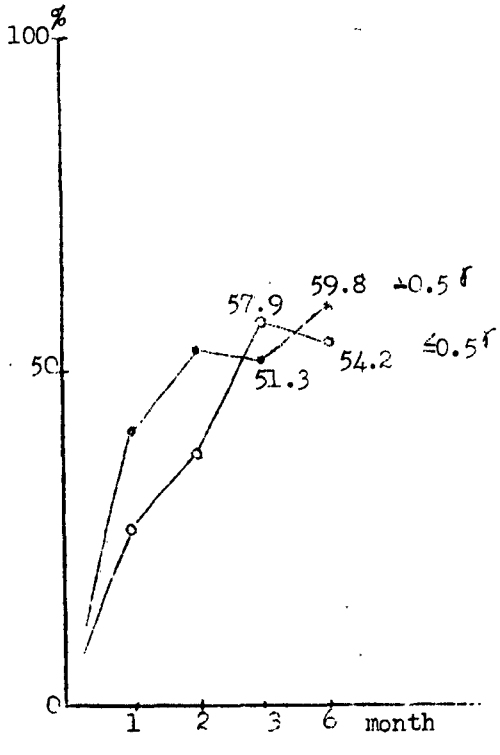
Fig. 11

Relationship between clinical effect of INH-Sulfonamide treatment and blood level 4 hours after dose of INH (127 subjects)

Comparability of different groups

Blood level		≤0.5γ	>0.5γ
Total number		49 (100%)	78 (100%)
Sex	Male	63.3	64.2
	Female	36.7	35.8
Age	-20	18.3	62.8
	21-50	65.5	64.1
	51-	16.2	23.1
NTA Classification			
Min.		8.1	5.1
Mod. adv		34.7	46.2
Far adv.		57.2	48.7

	≤0.5γ	>0.5γ
Noncavitary lesion		
Infiltrative caseous	85.9	82.1
Fibrocaseous	8.1	12.8
Far adv. mixed form	4.0	5.1
Others	2.0	
Cavity		
No cavity	22.4	21.0
Single cavity		
Sclerotic	8.2	5.1
Nonsclerotic	20.4	34.6
Multiple cavity		
Sclerotic	6.1	2.6
Fonsclerotic	42.9	36.7



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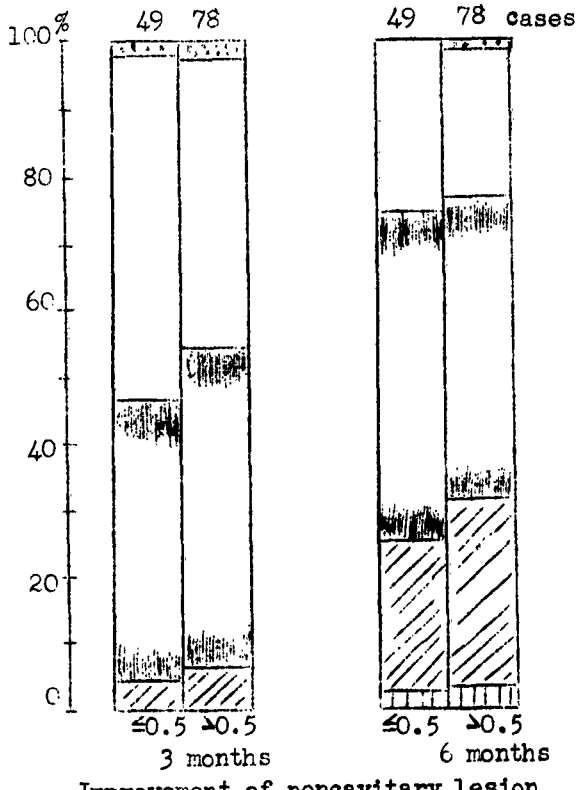


Improvement of noncavitary lesion

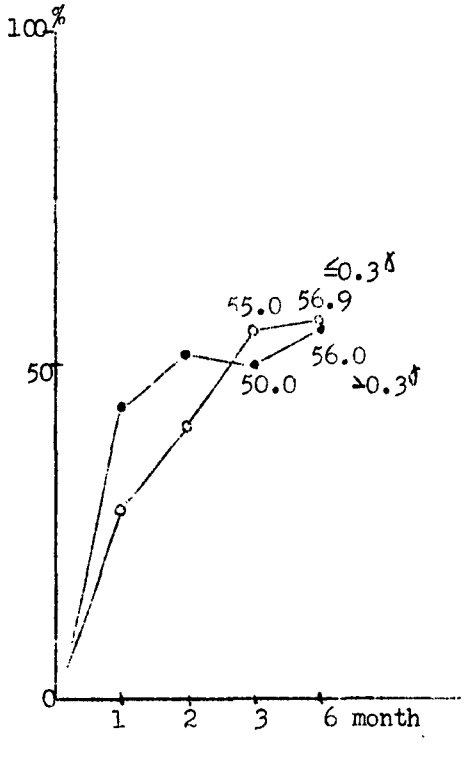
Fig.12

Relationship between clinical effect of INH-Sulfa treatment and blood level 6 hours after 4 mg/kg dose of INH (129 subjects)

Comparability of different groups

Blood level		≤0.3γ	>0.3γ
Total number		74 100%	55 100%
Sex	Male	64.8	61.8
	Female	35.2	38.2
Age	-20	14.8	10.8
	21-50	66.5	55.5
	51-	18.7	23.7
NTA Classification			
Min.		5.4	7.3
Mod. adv.		39.3	43.6
Far adv.		55.3	49.1

	≤0.3γ	>0.3γ
Noncavitary lesion		
Infiltrative caseous	83.9	81.9
Fibro caseous	6.7	16.3
Far adv. mixed form	8.1	1.8
Others	1.3	0
Cavity		
No cavity	20.3	23.6
Single cavity		
Sclerotic	5.4	7.3
Nonsclerotic	27.1	31.0
Multiple cavity		
Sclerotic	8.1	0
Nonsclerotic	39.1	38.1



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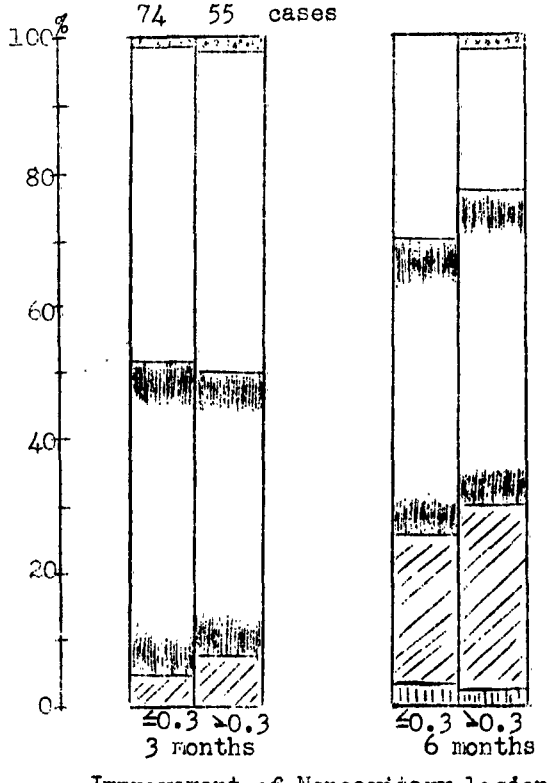


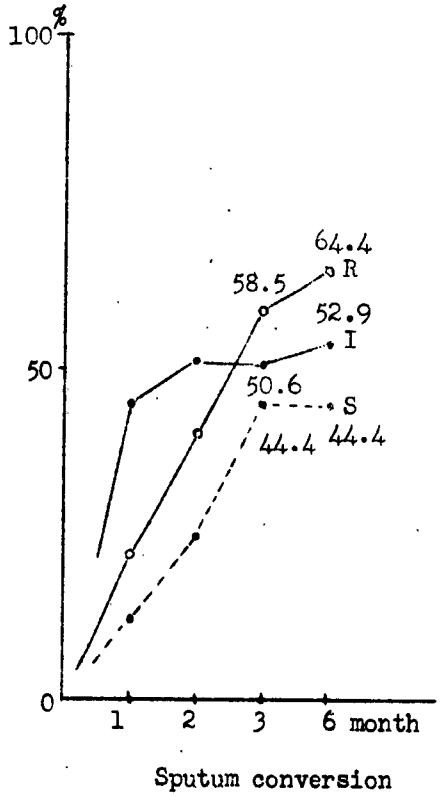
Improvement of Noncavitary lesion

Fig. 13

Relationship between clinical effect of INH-Sulfonamide treatment and pattern of INH inactivation (129 subjects)

Comparability of different groups

Pattern of INH inactivation		R	I	S
Total number		46 (100%)	74 (100%)	9 (100%)
Sex	Male	67.4	63.5	44.5
	Female	32.6	36.5	55.5
Age	-20	19.8	12.9	11.2
	21-50	67.4	64.1	66.6
	51-	12.8	23.0	22.2
NTA Classification				
Min.		8.7	4.1	11.2
Mod. adv.		37.0	45.9	22.2
Far adv.		54.3	50.0	66.6
Noncavitary lesion				
Infiltrative caseous		84.8	84.9	55.5
Fibro caseous		6.5	11.0	33.3
Far adv. mixed form		6.5	4.1	11.2
Others		2.2	0	0
Cavity				
No cavity		26.2	18.9	22.3
Single cavity				
Sclerotic		6.5	4.1	22.3
Nonsclerotic		22.0	35.1	11.2
Multiple cavity				
Sclerotic		2.2	6.8	0
Nonsclerotic		43.1	35.1	44.2



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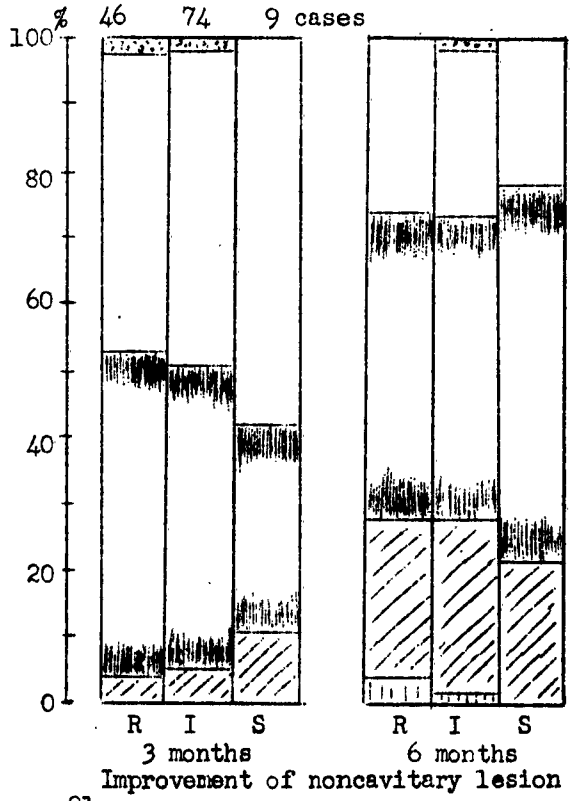


Improvement of noncavitary lesion

Fig. 14

Relationship between regression of nonsclerotic cavity in INH-Sulfa treatment and blood level after 4 mg/kg dose of INH (125 subjects)

Comparability of different groups

Cavity	Pattern of inactivation R	I	S	6 hr level ≦0.3	>0.3	4 hr level ≦0.5	>0.5
Round	24 (55.5%)	42 (58.4)	4 (44.2)	43 (56.3)	27 (55.1)	26 (55.1)	44 (57.9)
Irregular	20 (44.5%)	30 (41.6)	5 (55.8)	33 (43.7)	22 (44.9)	21 (44.9)	32 (42.1)

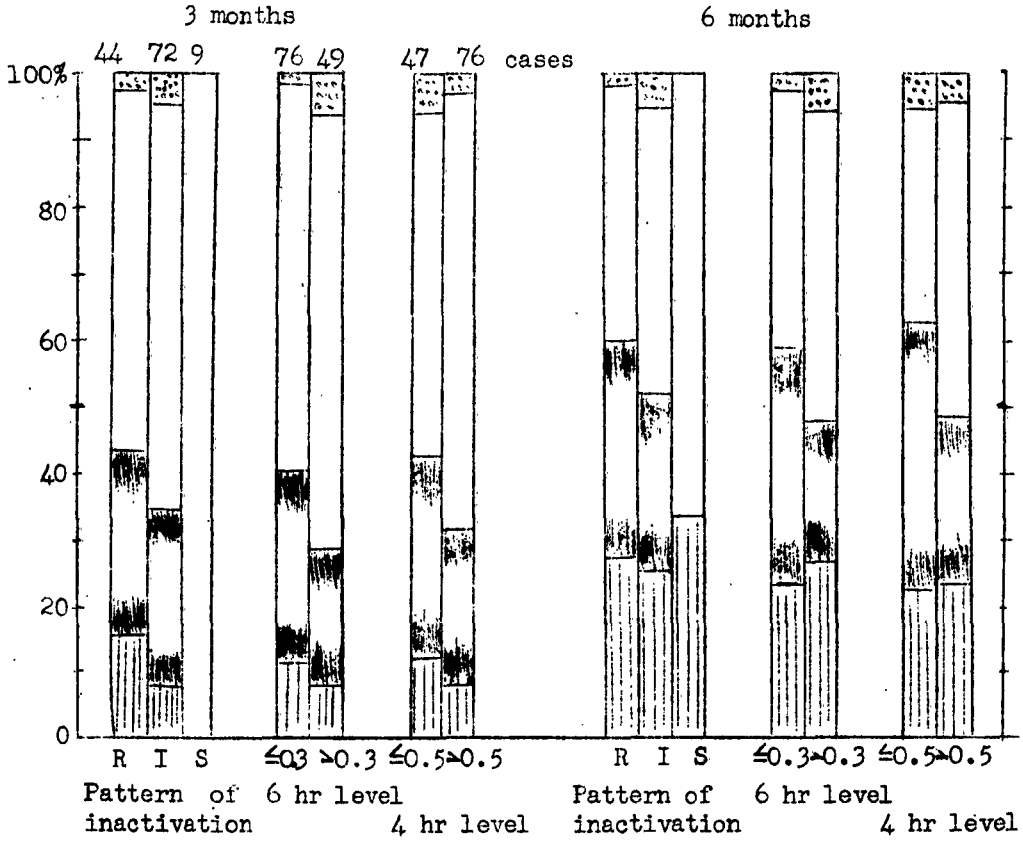


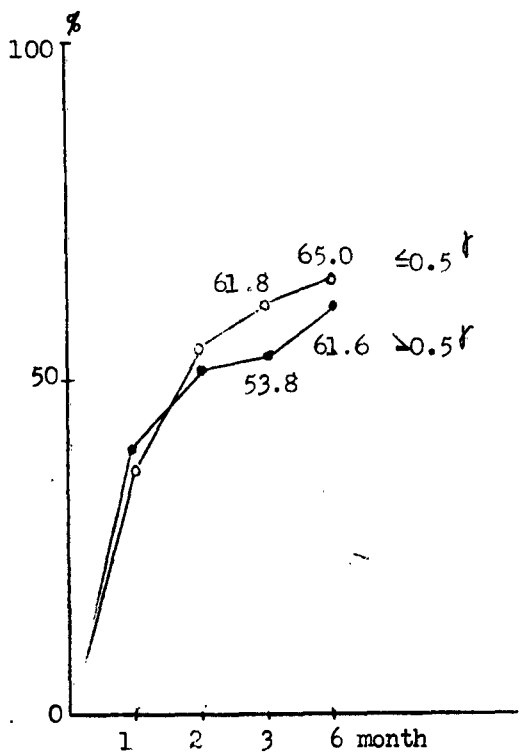
Fig. 15

Relationship between clinical effect of INH treatment (INH-Sulfa, INH-PZA & INH-PAS included) and 4 hour level after 4 mg/kg of INH (125 subjects)

Comparability of different groups

Blood level		≤0.5γ	>0.5γ
Total number		101	124
		100%	100%
Sex	Male	65.3	67.8
	Female	34.7	32.2
Age	-20	19.8	13.7
	21-50	61.4	63.7
	51-	18.8	22.6
NTA Classification			
Min.		8.9	8.1
Mod. adv.		37.6	42.0
Far adv.		53.5	49.9

	≤0.5γ	>0.5γ
Noncavitary lesion		
Infiltrative caseous	83.2	83.1
Fibro caseous	11.9	12.1
Far adv. mixed form	3.9	4.8
Others	1.0	
Cavity		
No cavity	18.8	16.9
Single cavity		
Sclerotic	7.9	4.8
Nonsclerotic	33.7	31.3
Multiple cavity		
Sclerotic	5.9	3.2
Nonsclerotic	33.7	43.8



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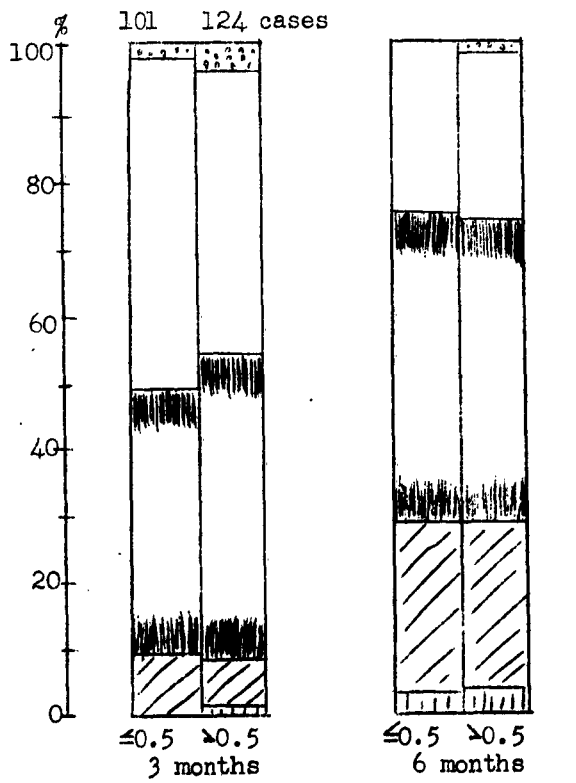


Improvement of noncavitary lesion

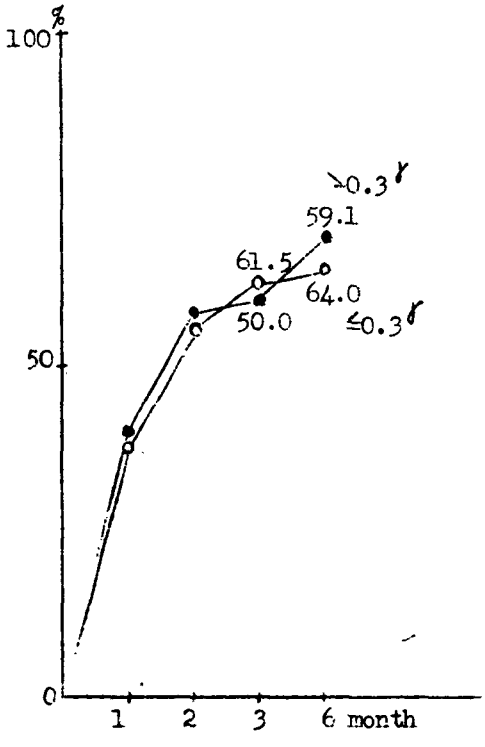
Fig. 16

Relationship between clinical improvement in INH treatment (INH-PAS, INH-PZA and INH-Sulfa included) and 6 hour level after 4 mg/kg of INH (233 subjects)

Comparability of different groups

Blood level		≤0.3γ	>0.3γ
Total number		138	95
		100%	100%
Sex	Male	66.6	68.5
	Female	33.4	31.5
Age	-20	18.8	13.7
	21-50	60.2	64.2
	51-	21.0	22.1
NTA Classification			
Min.		7.2	9.5
Mod. adv.		40.6	39.0
Far adv.		52.2	51.5

	≤0.3γ	>0.3γ
Noncavitary lesion		
Infiltrative caseous	81.6	84.1
Fibrocaseous	12.3	12.7
Far adv. mixed form	5.1	3.2
Others	1.0	0
Cavity		
No cavity	19.6	16.8
Single cavity		
Sclerotic	5.1	6.3
Nonsclerotic	33.4	29.4
Multiple cavity		
Sclerotic	7.2	6.3
Nonsclerotic	34.7	41.2



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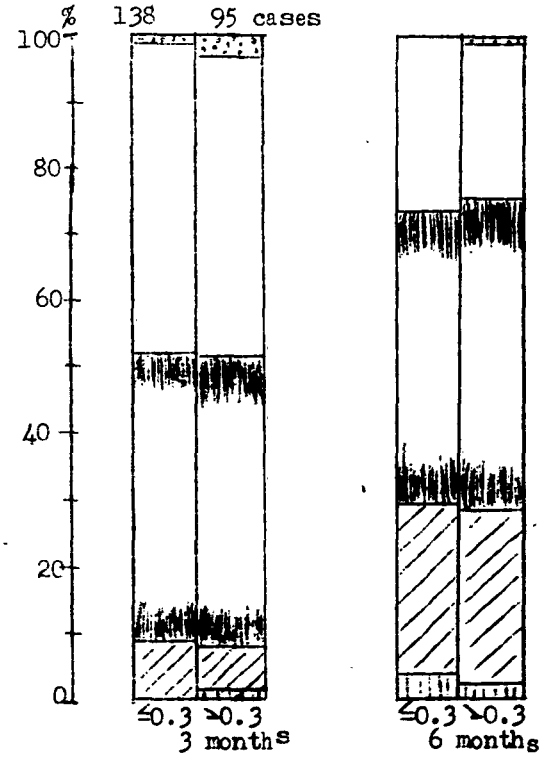


Improvement of noncavitary lesion

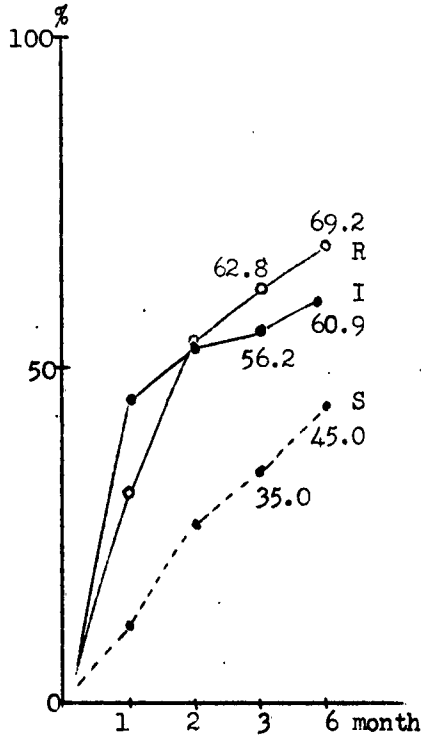
Fig. 17

Relationship between clinical improvement in INH treatment (INH-PAS, INH-PZA & INH-Sulfa included) and pattern of INH inactivation (233 subjects)

Comparability of different groups

Pattern of INH inactivation		R	I	S
Total number		88 100%	125 100%	20 100%
Sex	Male	67.1	67.9	65.0
	Female	32.9	32.1	35.0
Age	-20	20.6	15.3	10.0
	21-50	61.3	62.5	60.0
	51-	18.1	22.2	30.0
NTA Classification				
Min.		7.9	8.0	10.0
Mod. adv.		41.0	44.0	10.0
Far adv.		51.1	48.0	80.0

	R	I	S
Noncavitary lesion			
Infiltrative cas.	81.8	83.9	75.0
Fibrocaseous	12.5	12.1	15.0
Far adv. mixed	4.6	0	10.0
Others	1.1	4.0	0
Cavity			
No cavity	20.1	17.7	15.0
Single cavity			
Sclerotic	6.7	6.4	10.0
Nonsclerotic	35.3	33.5	5.0
Multiple cavity			
Sclerotic	3.4	8.0	15.0
Nonsclerotic	34.5	34.4	55.0



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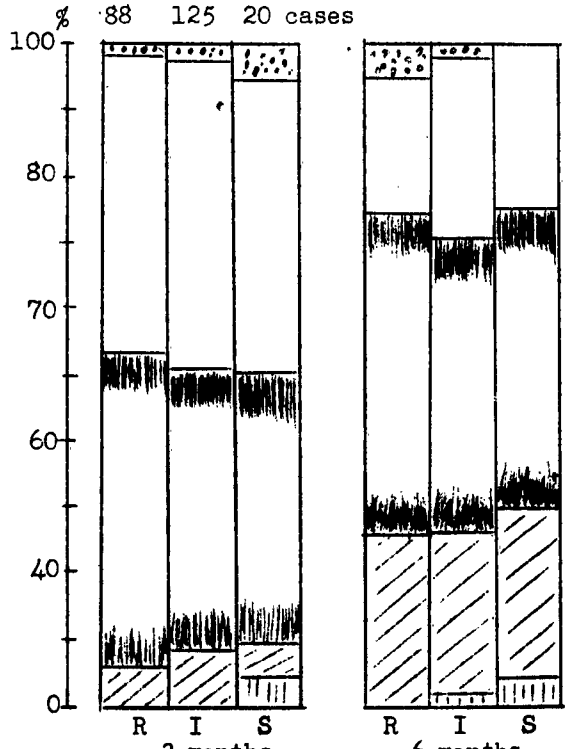


Improvement of noncavitary lesion

Fig. 18

Relationship between regression of nonsclerotic cavity in INH therapy (INH-PAS, INH-PZA & INH-Sulfa included) and blood level after 4 mg/kg dose of INH (225 subjects)

Comparability of different groups

Cavity	Pattern of inactivation R	I	S	6 hour level ≤0.3	>0.3	4 hour level ≤0.5	>0.5
Round	53 (64.6%)	68 (57.1)	8 (33.1)	81 (60.8)	48 (52.2)	55 (60.3)	72 (55.8)
Irregular	29 (35.4%)	51 (42.9)	16 (66.9)	52 (39.2)	44 (47.8)	36 (39.7)	57 (44.2)

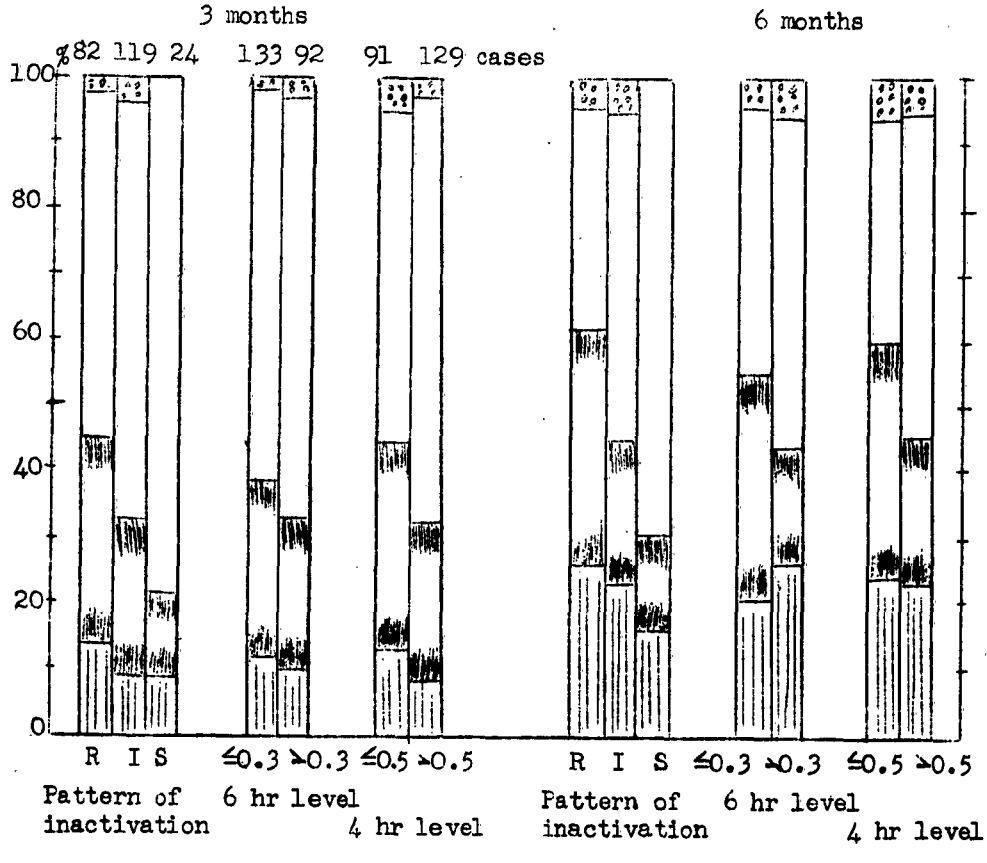


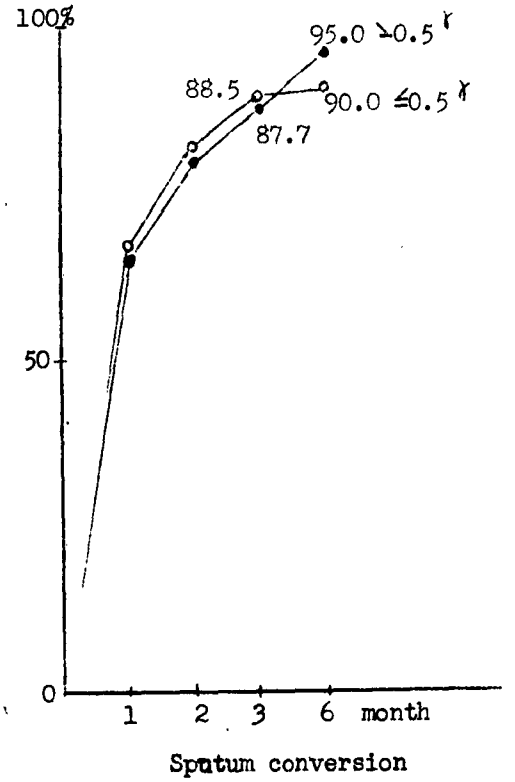
Fig. 19

Clinical effect of INH therapy (INH 0.2-0.3 g daily + PAS 10 g daily) related to 6 hour level after 4 mg/kg of INH (126 subjects)

Comparability of different groups

Blood level		≤0.5 γ	>0.5 γ
Total number		61	65
		100%	100%
Sex	Male	77.0	73.9
	Female	23.0	26.1
Age	-20	29.5	21.6
	21-50	60.6	70.8
	51-	6.6	4.6
	Unknown	3.3	3.0
NTA Classification			
Min		28.0	32.3
Mod. adv		60.6	52.3
Far adv.		11.4	15.4

		≤0.5 γ	>0.5 γ
Noncavitary lesion			
Infiltrative caseous		80.3	92.4
Fibro caseous		14.8	4.5
Far adv. mixed form		0	0
Others		4.9	3.1
Cavity			
No cavity		47.5	49.2
Single cavity			
Sclerotic		0	0
Nonsclerotic		42.6	24.7
Multiple cavity			
Sclerotic		0	0
Nonsclerotic		9.8	23.1
Unknown		0	3.0
Pattern	R	75.5	12.2
	I	22.9	63.1
	S	0	24.7
	Unknown	1.6	0



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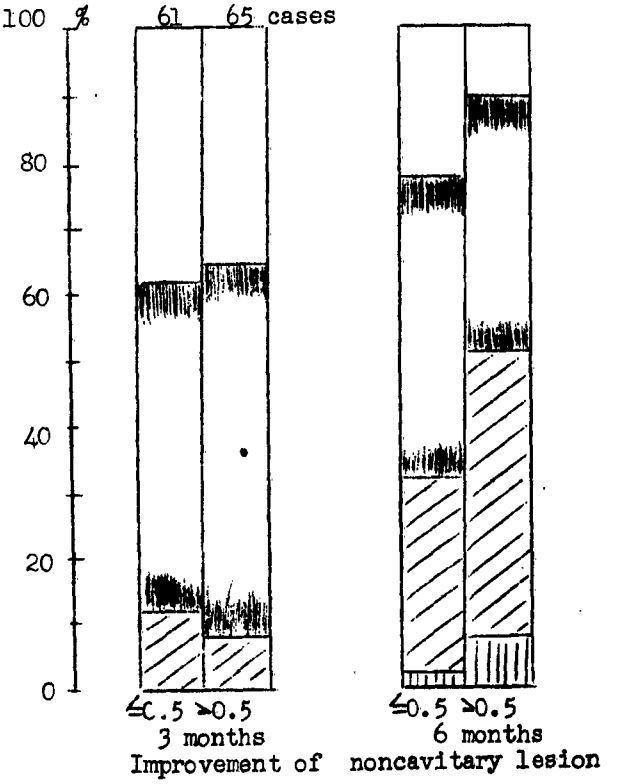


Improvement of noncavitary lesion

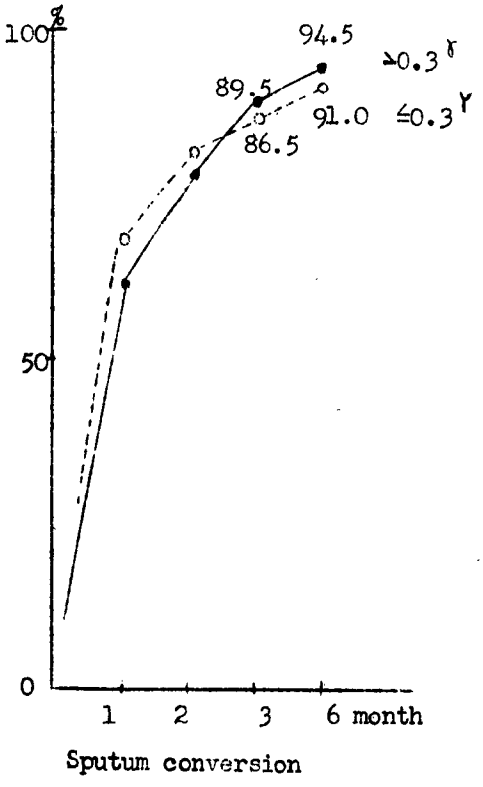
Fig. 20

Clinical effect of INH therapy (INH 0.2-0.3 g daily + PAS 10 g daily) related to 6 hour level after 4 mg/kg of INH (125 subjects)

Comparability of different groups

		≦0.3 γ	≧0.3 γ
Blood level			
Total number		68	57
		100%	100%
Sex	Male	72.0	79.0
	Female	28.0	21.0
Age	-20	31.0	19.3
	21-50	60.2	72.0
	51-	5.9	5.2
	Unknown	2.9	3.5
NTA Classification			
Min.		31.0	29.9
Mod. adv.		57.5	54.5
Far adv.		11.5	15.6

		≦0.3 γ	≧0.3 γ
Noncavitary lesion			
Infiltrative caseous		81.0	93.0
Fibrocaseous		14.6	3.5
Far adv. mixed form		0	0
Others		4.4	3.5
Cavity			
No cavity		50.0	45.6
Single cavity			
Sclerotic		0	0
Nonsclerotic		38.5	28.2
Multiple cavity			
Sclerotic		0	0
Nonsclerotic		11.5	22.7
Unknown		0	3.5
Pattern	R	79.0	0
	I	21.0	72.0
	S	0	28.0



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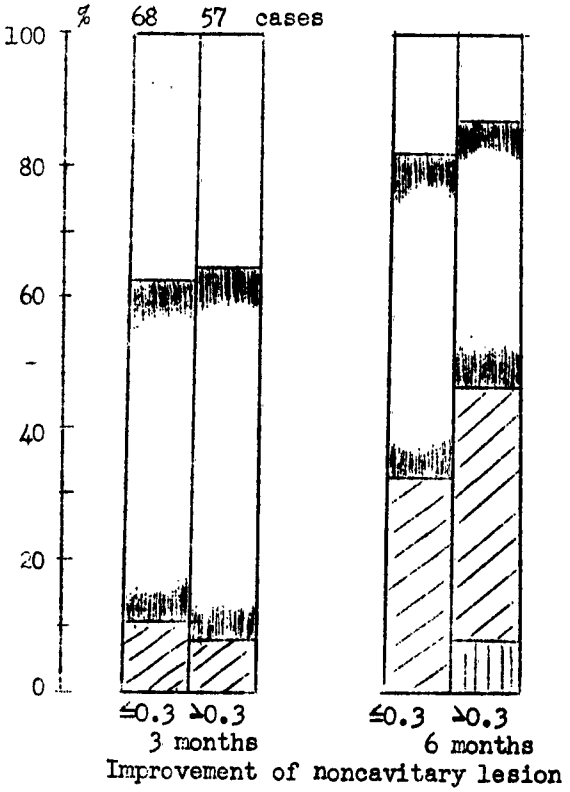


Improvement of noncavitary lesion

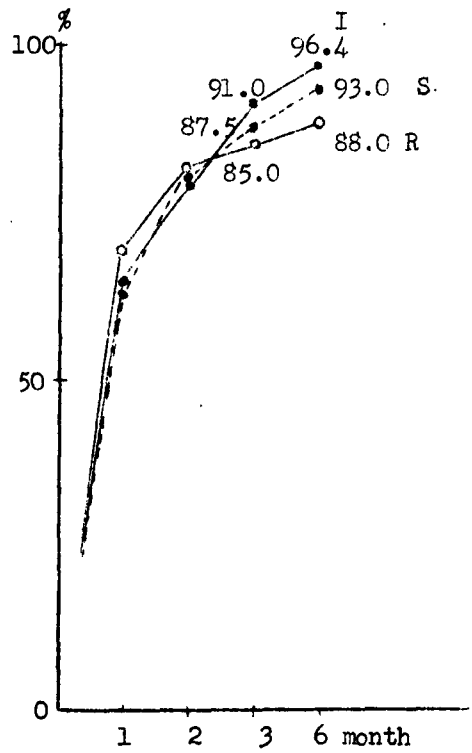
Fig. 21

Clinical effect of ordinary dose INH therapy (INH 0.2-0.3 g daily + PAS 10 g daily) related to pattern of INH metabolism after 4 mg/kg dose of INH (125 subjects)

Comparability of different groups

Pattern of INH inactivation		R	I	S
Total number		54 100%	55 100%	16 100%
Sex	Male	72.2	74.5	87.5
	Female	27.8	25.5	12.5
Age	-20	24.0	29.1	18.8
	21-50	66.8	69.1	50.0
	21-	5.5	1.8	18.8
	Unknown	3.7	0	12.4
NTA Classification				
Min.		31.4	33.6	50.0
Mod. adv.		55.7	62.0	37.5
Far adv.		12.9	24.4	12.5

	R	I	S
Noncavitary lesion			
Infiltrative cas.	81.8	91.0	87.6
Fibrocaseous	14.5	5.4	6.2
Far adv. mixed form	0	0	0
Others	3.7	3.6	6.2
Cavity			
No cavity	46.2	49.1	50.0
Single cavity			
Sclerotic	0	0	0
Nonsclerotic	40.9	29.1	25.0
Multiple cavity			
Sclerotic	0	0	0
Nonsclerotic	12.9	18.2	25.0
Unknown	0	3.6	0



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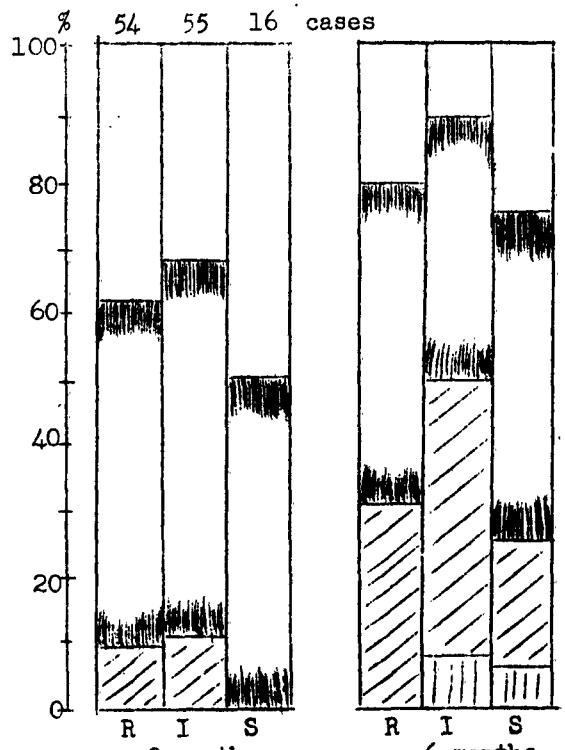


Improvement of noncavitary lesion

Fig. 22

Improvement of cavity in ordinary dose INH therapy (INH 0.2-0.3 g + PAS 10 g daily) related to pattern of INH inactivation, 4 and 6 hour levels after 4 mg/kg dose of INH (63 subjects)

	Comparability of different groups						
	Pattern of inactivation			6 hour level		4 hour level	
	R	I	S	≤0.3 γ	>0.3 γ	≤0.5 γ	>0.5 γ
Total number	29 100%	26 100%	8 100%	34 100%	29 100%	32 100%	31 100%
Single cavity case	76.0	61.5	50.0	76.2	55.2	81.1	51.4
Multiple cavity case	24.0	38.5	50.0	23.8	44.8	18.9	48.6

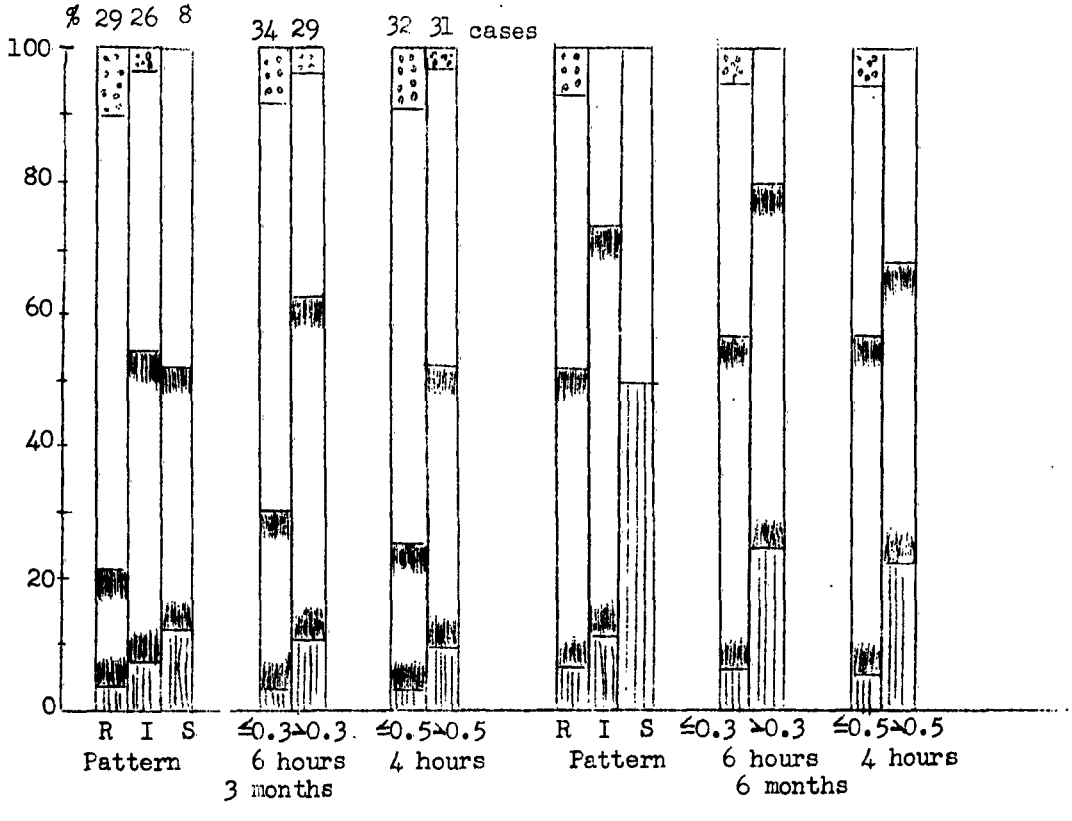


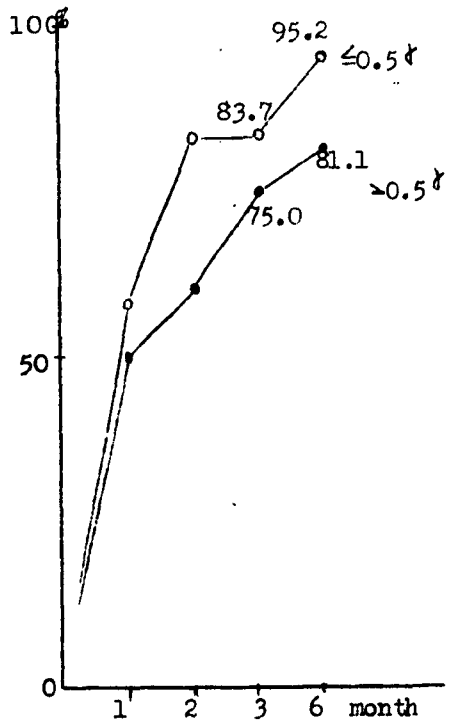
Fig. 23

Clinical effect of ordinary and high dose INH-PAS therapy related to 4 hour blood level after actually prescribed single dose (85 subjects)

Comparatibility of different groups

Blood level		≤0.5 γ	>0.5 γ
Total number		43 100%	42 100%
Sex	Male	67.4	71.4
	Female	32.6	28.6
Age	-20	32.6	23.8
	21-50	60.5	62.0
	51-	4.6	14.2
	Unknown	2.3	0
NTA Classification			
Min.		16.3	19.0
Mod. adv.		60.5	50.0
Far adv.		23.2	31.0
Noncavitary lesion			
Infiltrative caseous		86.1	85.7
Fibro caseous		7.0	9.5
Far adv. mixed form		2.3	0
Others		4.6	4.8

	≤0.5 γ	>0.5 γ
Cavity		
No cavity	37.2	28.5
Single cavity		
Sclerotic	4.6	0
Nonsclerotic	32.6	31.0
Multiple cavity		
Sclerotic	0	2.4
Nonsclerotic	18.6	35.7
Unknown	7.0	2.4
Dose of INH		
INH 18 mg/kg	16.3	54.9
INH 0.2-0.3 g	83.7	45.1
Metabolic pattern		
R	48.8	35.7
I	48.8	40.5
S	2.4	23.8



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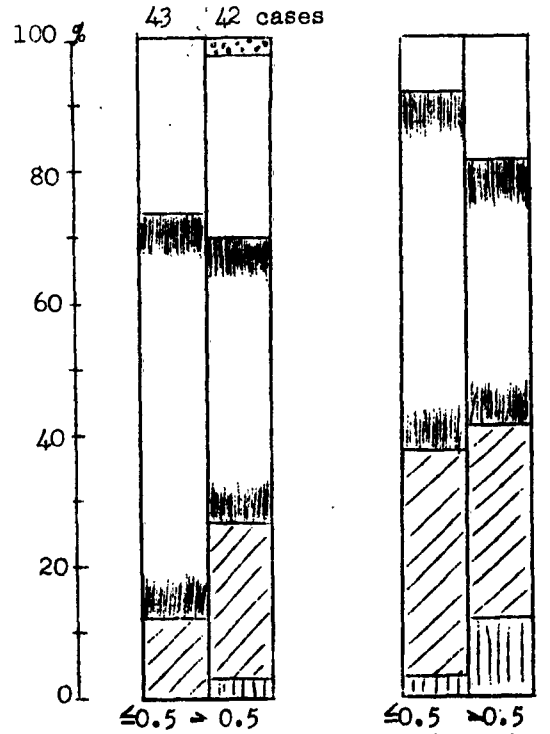


Improvement of noncavitary lesion

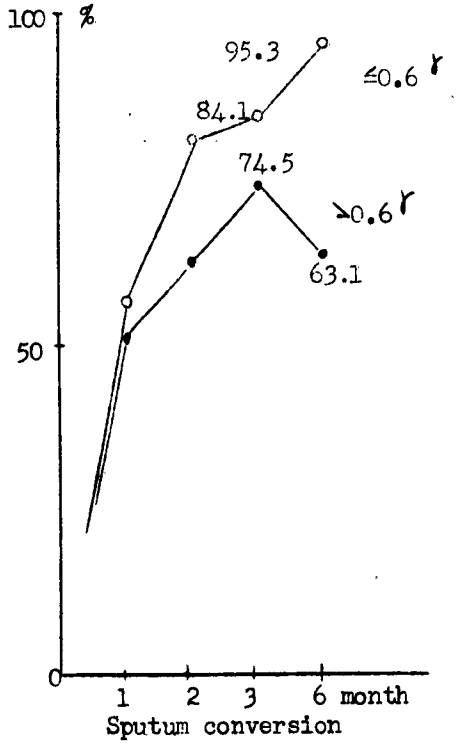
Fig. 24

Clinical effect of ordinary and high dose INH-PAS therapy related to the average concentration at 2, 4 and 6 hours after actual dose (85 subjects)

Comparability of different groups

Blood level		≤0.6 γ	>0.6 γ
Total number		44 100%	41 100%
Sex	Male	68.1	70.8
	Female	31.9	29.2
Age	-20	34.0	21.9
	21-50	59.2	63.5
	51-	4.5	14.6
	Unknown	2.3	
NTA Classification			
Min.		18.1	17.1
Mod. adv.		59.2	51.3
Far adv.		22.7	31.6
Noncavitary lesion			
Infiltrative caseous		88.6	83.0
Fibro caseous		6.8	9.8
Far adv. mixed form		0	2.4
Others		4.6	4.8

		≤0.6 γ	>0.6 γ
Cavity			
No cavity		36.5	29.3
Single cavity			
Sclerotic		2.3	2.4
Nonsclerotic		34.0	29.3
Multiple cavity			
Sclerotic		0	2.4
Nonsclerotic		20.4	34.2
Unknown		6.8	2.4
Dose of INH			
INH 18 mg/kg		13.4	58.6
INH 0.2-0.3 g		86.6	41.4
Metabolic pattern			
R		45.5	39.1
I		52.2	36.5
S		2.3	24.4



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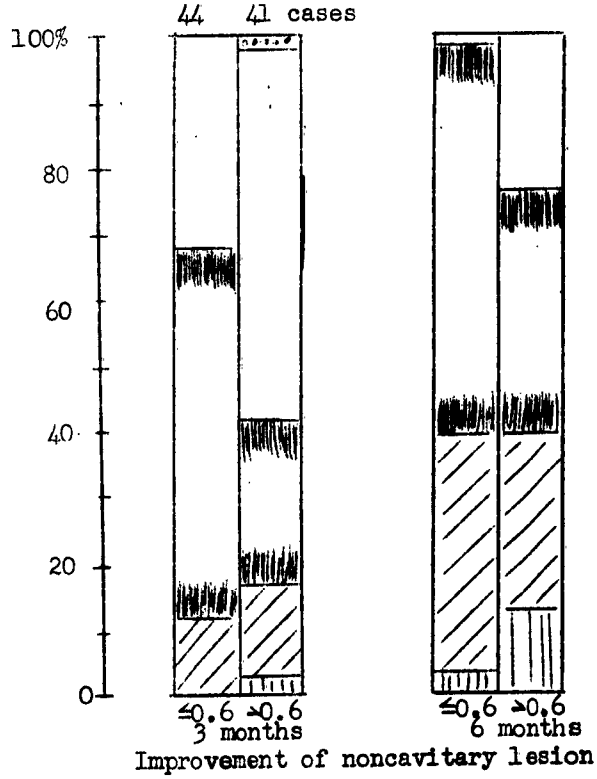


Improvement of noncavitary lesion

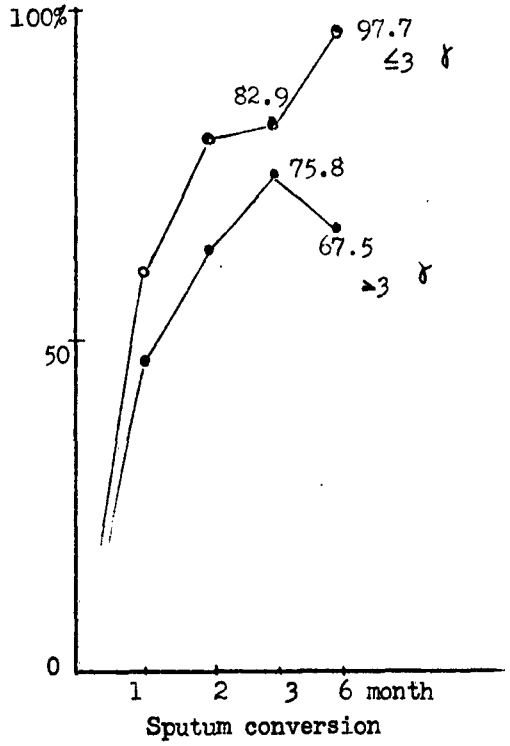
Fig. 25

Clinical effect of ordinary and high dose INH-PAS therapy related to the area of time concentration curve after actually prescribed single dose (85 subjects)

Comparability of different groups

Blood level		≤3 γ	>3 γ
Total number		41 100%	44 100%
Sex	Male	70.8	68.1
	Female	29.2	31.9
Age	-20	34.2	22.7
	21-50	58.6	63.9
	51-	4.8	13.4
	Unknown	2.4	
NTA Classification			
Min.		19.5	34.0
Mod. adv.		61.0	50.0
Far adv.		19.5	16.0
Noncavitary lesion			
Infiltrative caseous		88.0	84.0
Fibro caseous		7.2	9.1
Far adv. mixed form		0	2.3
Others		4.8	4.6

	≤3 γ	>3 γ
Cavity		
No cavity	36.6	29.5
Single cavity		
Sclerotic	2.4	2.3
Nonsclerotic	31.7	31.8
Multiple cavity		
Sclerotic	0	2.3
Nonsclerotic	21.9	31.8
Unknown	7.4	2.3
Dose of INH		
INH 18 mg/kg	7.2	61.5
INH 0.2-0.3 g	92.8	38.5
Metabolic pattern		
R	46.4	38.7
I	51.2	38.7
S	2.4	22.6



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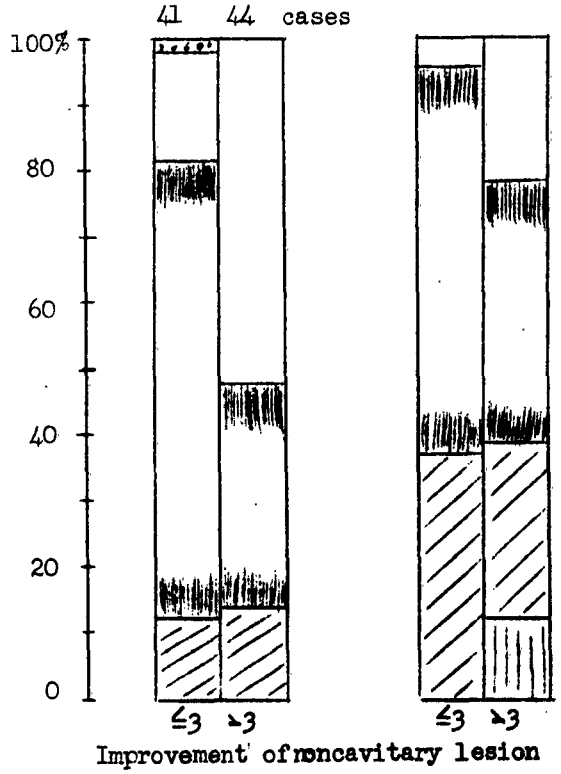


Improvement of noncavitary lesion

3 months 6 months

Fig. 26

Change of individual cavities related to blood level after actual dose (ordinary and high dose INH-PAS therapy) (70 subjects)

	Blood level	Total number	Comparability: Nonsclerotic cavity	Comparability: Sclerotic cavity
4 hour level	≤0.5 γ	26	24 (92.2%)	2 (7.8%)
	>0.5	44	43 (97.7%)	1 (2.3%)
Average of 2,	≤0.6	27	26 (96.3%)	1 (3.7%)
4 & 6 hr levels	>0.6	43	41 (95.4%)	2 (4.6%)
Area of time	≤3	24	23 (95.8%)	1 (4.2%)
concentration curve	>3	46	44 (95.7%)	2 (4.3%)

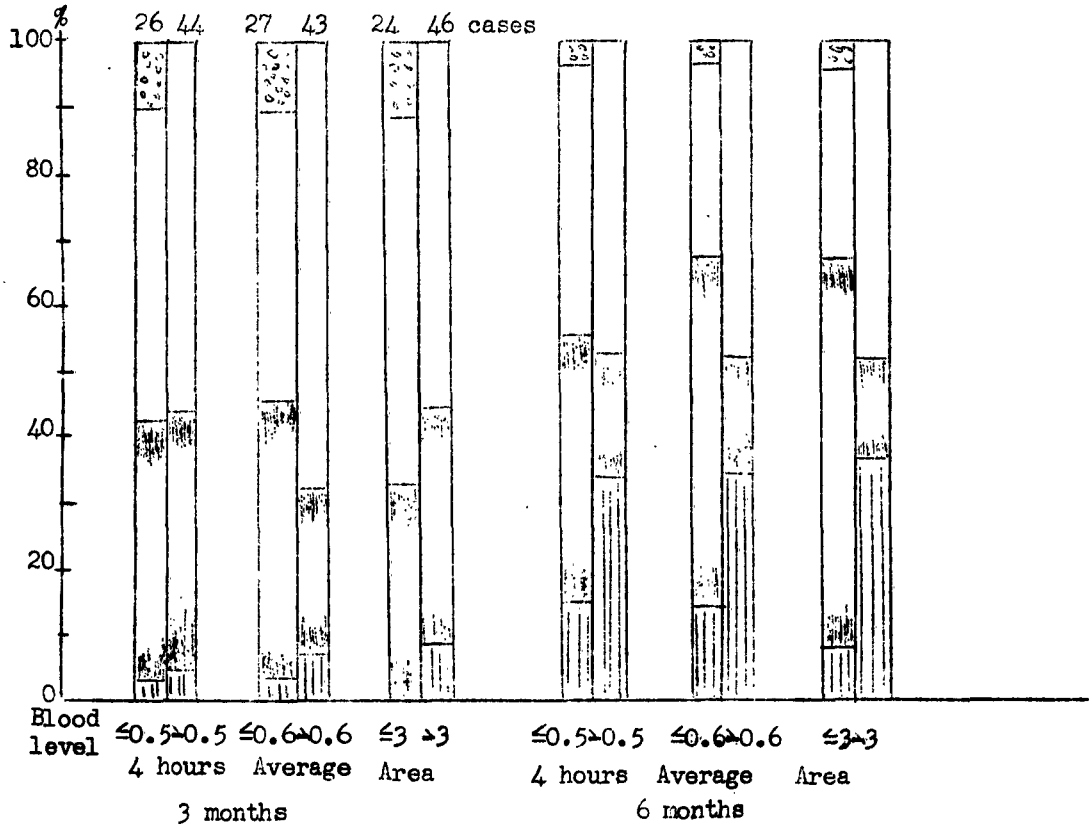


Fig. 27

Cavity change related to blood level of biologically active INH after administration of actually prescribed dose of INH (cavities found in a subject are treated together) (ordinary and high dose INH-PAS therapy) (53 subjects)

	Comparability					
	4 hour level		Average		Area	
	≤0.5γ	>0.5γ	≤0.6γ	>0.6γ	≤3.0γ	>3.0γ
Total number	24	29	25	28	23	30
	100%	100%	100%	100%	100%	100%
Single cavity case						
Sclerotic	8.3	0	4.0	3.6	4.4	3.3
Nonsclerotic	58.4	14.9	60.0	42.8	56.4	46.7
Multiple cavity case						
Sclerotic	0	3.4	0	3.6	0	3.3
Nonsclerotic	33.3	51.9	36.0	50.0	39.2	46.7

Average: average of 2, 4 & 6 hours' concentrations
Area: area of time concentration curve

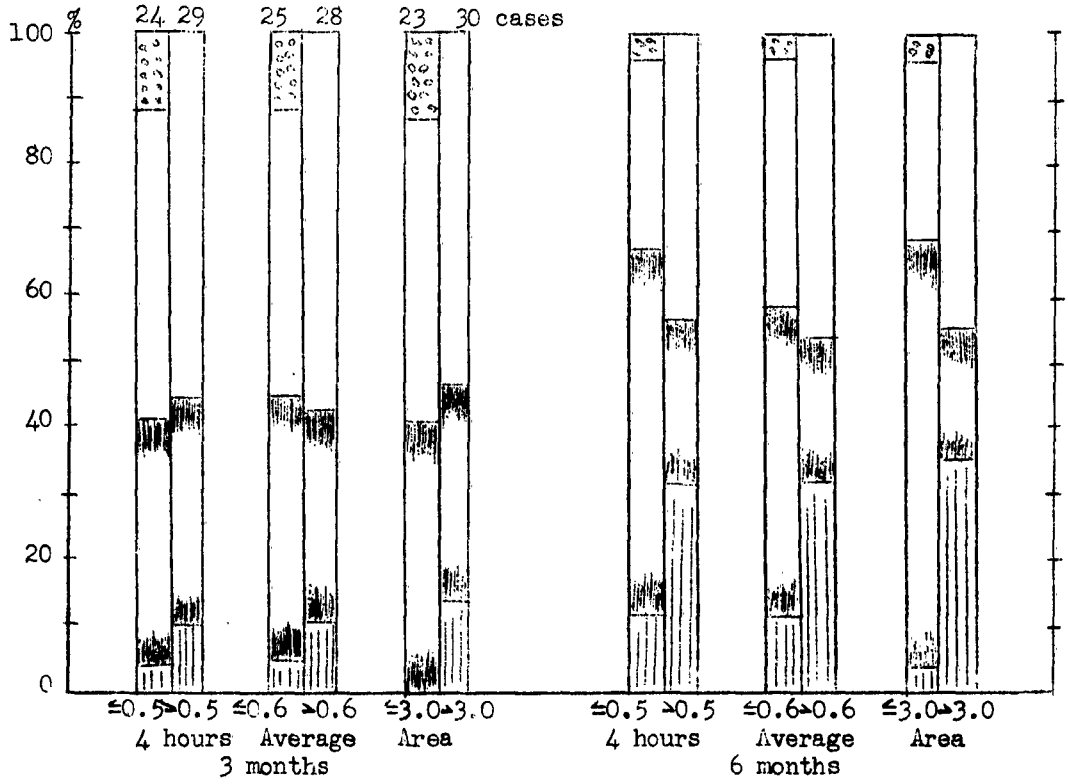


Fig. 28

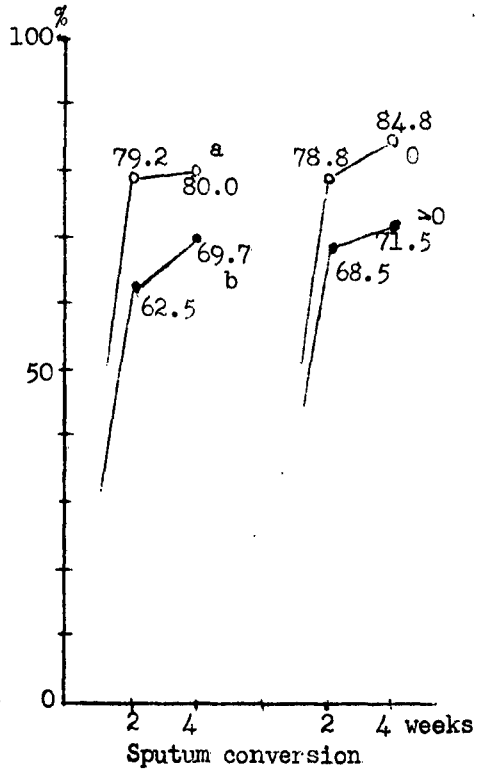
Comparison of therapeutic effect between ordinary and very low dose of INH combined with PAS (51 subjects)

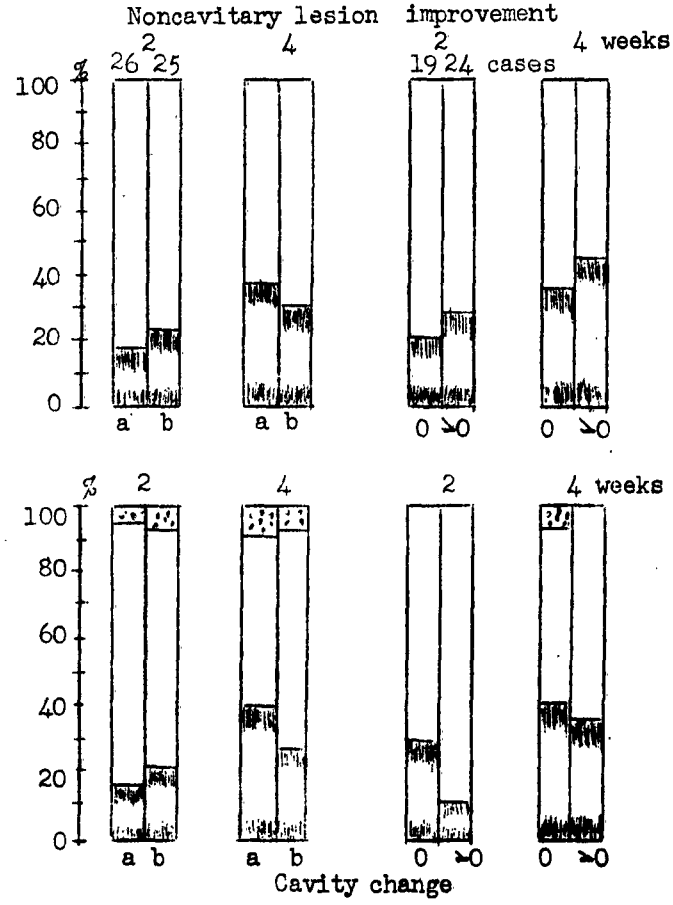
a: cases treated with 2 mg/kg INH in two divided doses, & b: with 4 mg/kg

0: no biologically active INH in 4 hours' blood, & >0: biologically active INH was measured

Comparability

		a	b	0	>0
Total number		26	25	19	24
		100%	100%	100%	100%
Sex	Male	73.2	60.0	63.0	67.0
	Female	26.8	40.0	37.0	33.0
Age	-20	34.7	12.0	26.4	12.5
	21-50	53.8	56.0	63.0	62.5
	51-	3.8	32.0	10.6	20.8
	Unknown	7.7	0	0	4.2
NTA Classification					
Min.		19.2	20.0	21.2	16.6
Mod. adv.		69.3	60.0	15.8	66.8
Far adv.		11.5	20.0	63.0	16.6
Noncavitary lesion					
Infiltrative caseous		96.2	88.0	89.4	91.6
Fibro caseous		3.8	12.0	10.6	8.4
Cavity					
No cavity		37.4	44.0	37.0	50.0
Single cavity					
Sclerotic		0	4.0	5.2	0
Nonsclerotic		57.6	28.0	47.4	20.8
Multiple cavity					
Sclerotic		0	4.0	0	4.2
Nonsclerotic		7.7	20.0	10.4	25.0
Metabolic pattern					
R		53.8	76.0	63.0	70.8
I		30.9	12.0	21.2	25.0
S		3.8			4.2
Unknown		11.5	12.0	15.8	0





3. BIOCHEMICAL STUDIES ON ACETYLATION IN ANIMAL TISSUE

We intended to reveal the cause and mechanism of individual and species difference with respect to isoniazid inactivation and carried out the following animal experiments.

a. Acetylation of isoniazid in liver homogenate of various species of animals

As shown in Fig. 29, acetylating activity of chicken liver homogenate was the most active, and followed by rabbit, guinea pig and rat liver in the indicated order. In the case of rat liver, the activity was almost negligible. It was concluded that there was a definite species difference of isoniazid metabolism. Addition of acetate (0.04 mM) to the reaction system enhanced acetylation remarkably in chicken and rabbit but no rise in the activity was observed in guinea pig and rat which almost lacked the ability to acetylate isoniazid by nature.

b. Acetylation of isoniazid by homogenate of various kinds of chicken organs

As shown in Fig. 30, the liver is the site of the most active acetylation, and followed by the kidney. In the lung, muscle and blood, the rate of acetylation was fairly small and negligible.

Relationship between oxidation and acetylation of isoniazid in several organs is illustrated in Fig. 31. In most of organs, acetylation was proportional to oxidation, the quotient: acetylation/oxidation being nearly 0.1, while in the liver, efficiency of acetylation was doubled as compared with other organs.

c. Acetylation of sulfonamide and paraaminobenzoic acid (PABA) by liver homogenate of various species of animals

As we revealed that the isoniazid acetylating ability of liver homogenate varied considerably from animal to animal, we tried to investigate whether it would be true also with other kinds of acetyl-acceptors such as sulfonamide and PABA. The experimental conditions were almost same as in the study on acetylation of isoniazid but the incubation time was raised to 60 minutes in the following experiment.

It is interesting to note in Fig. 32 that chicken liver homogenate acetylates sulfisoxazole to the least extent and the activity is definitely inferior to that of rabbit and guinea pig, while the exact reverse is the case with isoniazid.

The PABA acetylating activity of chicken liver homogenate was also insignificant contrary to our expectation as demonstrated in Fig. 33.

It is strange that acetylation of sulfisoxazole and PABA by chicken liver was the weakest or the second weakest in the above mentioned experiment, for high activity of pigeon liver homogenate with regard to acetylation of sulfanilamide and PABA was reported again and again by Lipmann (5) and other investigators (6). The discrepancy between these reports and ours must be due either to the difference in kinds of experimental animals such as chicken and pigeon or to the difference in kinds of sulfa-drugs such as sulfanilamide and sulfisoxazole.

In the first place, we investigated acetylation of sulfisoxazole by pigeon liver homogenate and compared it with the result by chicken liver homogenate. As shown in Fig. 34, it was revealed that sulfisoxazole was very difficult to acetylate even in pigeon liver, while the rate of PABA acetylation was much higher (about 65%) in pigeon liver than in chicken liver.

This observation supports the view that the reason for the low rate of acetylation of sulfonamide by chicken liver homogenate in our previous experiment is not to find in animal species (chicken) but in chemical structure of sulfonamide (sulfisoxazole).

Fig. 35 demonstrates clearly that pigeon liver homogenate acetylates sulfanilamide to the almost same degree as isoniazid in the sharp contrast to sulfisoxazole which remains nearly unacetylated.

In the case of chicken liver homogenate, the rates of acetylation of isoniazid and sulfanilamide were a little lower than in pigeon liver as indicated in Fig. 36.

It seems probable that there are both species and drug difference in the mechanism of acetylation: chicken differs not only from rabbit, guinea pig and rat but also from pigeon, and sulfanilamide differs not only from isoniazid and PABA but also from sulfisoxazole.

d. Relationship between acetylation of isoniazid and oxidation in the liver of several kinds of animals and influence of liver damage on acetylation

Acetylating activity of isoniazid of liver homogenate decreased in the order of pigeon, chicken, rabbit, guinea pig and rat as shown in Fig. 37, and it was not related to oxidation (Fig. 37 and 38).

As it was established that the liver was the most important organ with regard to acetylation, we intended to observe a change in acetylation in experimentally damaged chicken liver. We injected CCl_4 in the dose of 1 ml/kg body weight to chickens intramuscularly for two consecutive days and sacrificed them on the third day. Although the dose of CCl_4 used was considerably high, we did not observe any change in O_2 uptake but acetylating activity for isoniazid (Fig. 39) and sulfonamide (Fig. 40) decreased remarkably.

e. Intracellular localization of isoniazid acetylating principle

Chicken liver cell homogenate was prepared with isotonic sucrose solution, centrifuged by the Spinco ultra centrifuge and nuclear, microsomal, and supernatant fractions were separated by the Schneider's method. All the procedures were undertaken in low temperature. In the presence of sufficient amount of ATP, Mg^{++}, Co enzyme A, K^{+}, phosphate and acetate, isoniazid was incubated with the above mentioned fractions at 37° C. for 60 minutes in the Warburg's apparatus. After the reaction was halted, isoniazid acetylated was measured and expressed in γ/mg N.

Based on the data shown in Fig. 41, it was concluded that the enzymatic principle which brought out acetylation of isoniazid was bound neither to nucleus nor to mitochondria but to microsome and supernatant fluid. Especially supernatant fraction demonstrated the highest activity. It appears most likely that enzymatic principle is localized solely in fluid fraction and contamination during the preparation or insufficient fractionation results in some microsomal activity.

f. Effect of various kinds of substrates on acetylation of isoniazid.

Fig. 42 demonstrates the diagram of carbohydrate metabolism. We investigated the influence of the addition of acetate, pyruvate, α-ketoglutarate, fumarate, succinate, fructose and glucose to the reaction system on acetylation of isoniazid.

Effect of the increased dose of acetate on acetylation was indicated in Fig 43 and the comparison of the effects among various kinds of substrates added to the reaction system were illustrated in Fig. 44.

It was shown that substances located outside of TCA cycle like glucose, fructose, acetic acid and pyruvic acid more or less accelerated acetylation of isoniazid, while substances located within TCA cycle like fumaric acid, α-ketoglutaric acid and succinic acid had no effect on the rate of inactivation of isoniazid. It is note worthy that among the former group of substrates, acetic and pyruvic acid surpasses in this respect other substances whose positions shown in Fig. 42 are far from acetyl Co enzyme A.

g. Effect of Co enzyme A on acetylation and species difference in Co enzyme A content of the liver

We failed to increase the rate of acetylation of isoniazid by the addition of acetate to rat liver homogenate which nearly lacked the activity by nature as shown elsewhere and also in Fig.45.

In the following experiment, at first the role of Co enzyme A in acetylation in various species of animals was investigated. Definite increase of the rate of acetylation was observed after the addition of 50 or 100 units of purified Co enzyme A to rat liver homogenate, but the rate was only about 10% even in that case, while 50% of isoniazid was acetylated by pigeon or chicken liver homogenate even when no Co enzyme A was added to the reaction system.

It was established that the species difference in isoniazid inactivation between rat and pigeon or chicken could not be overcome by the addition of Co enzyme A.

We tested the influence of the addition of Co enzyme A to pigeon liver homogenate which had very high acetylation activity as shown in Fig. 46. In this experiment, the dose of isoniazid was raised to 400 mg and the amount of liver homogenate was reduced to half that in the preceeding experiment on rat liver, for a larger amount of Co enzyme A caused too sharp a rise in the rate of acetylation that the activity could not be measured precisely in our experimental condition mentioned above.

Co enzyme A content of the liver of chicken, pigeon, rabbit and rat was determined by Lipmann-Kaplan's method (2). At first, a standard curve for Co enzyme A as shown in Fig. 47 was drawn .

Though potency of the enzyme preparation in the first experiment (No. 1) was lower than that in the second experiment (No.2), if we defined the amount that activated each system to half the maximum activity as 1 Unit Co enzyme A according to Kaplan and Lipmann, the unit was largely independent of the individual preparation, even if the absolute amount of acetylated sulfanilamide might vary.

The practical saturation point was fixed at about 3 units and further addition of Co. enzyme A preparation to the reaction system was of almost no effect on the activity. By means of the standard curve, Co enzyme A content of unknown sample was determined.

Almost no difference was revealed among various species of animals some of which inactivated isoniazid very rapidly and some showed nearly no ability for isoniazid inactivation as indicated in Fig. 48.

Fig.29 INH acetylating activity of liver homogenate

Comparison of various species of animals and effect of added acetate

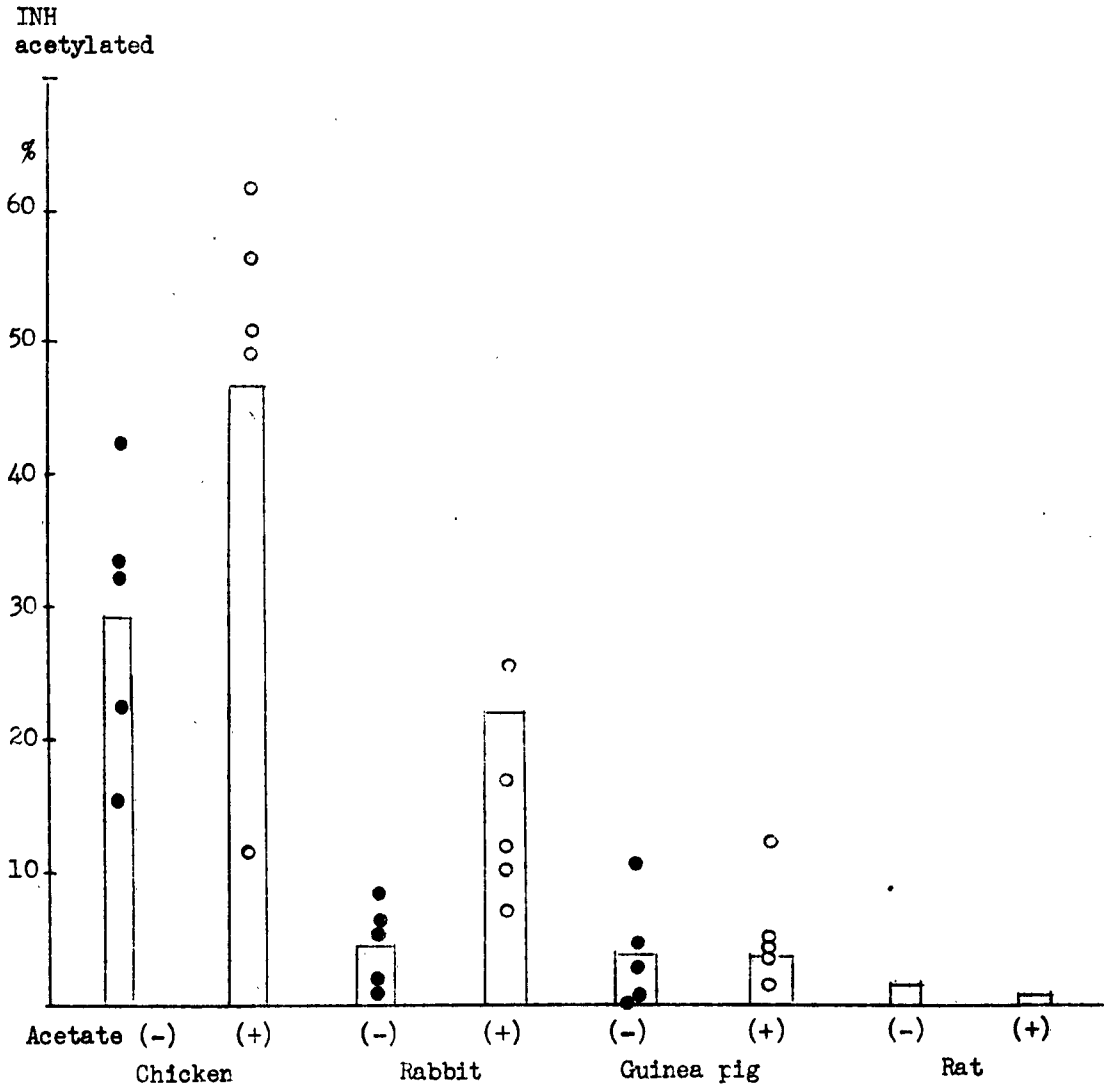


Fig. 30 INH acetylation by homogenate of various kinds of chicken organs

Organ	Exp. No. 1	No. 2	No. 3	No. 4	No. 5	Average
Blood	1.1%	3.6%	0.5%	3.4%	5.9%	2.9%
Kidney	19.2	21.5	19.1	13.2	8.4	16.5
Muscle	0.0	4.6	0.0	0.0	14.1	3.8
Brain	12.2	3.6	6.1	3.3	16.4	8.3
Liver	40.0	43.1	35.8	36.5	31.8	37.4
Lung	/	10.6	0.0	0.0	11.3	5.5

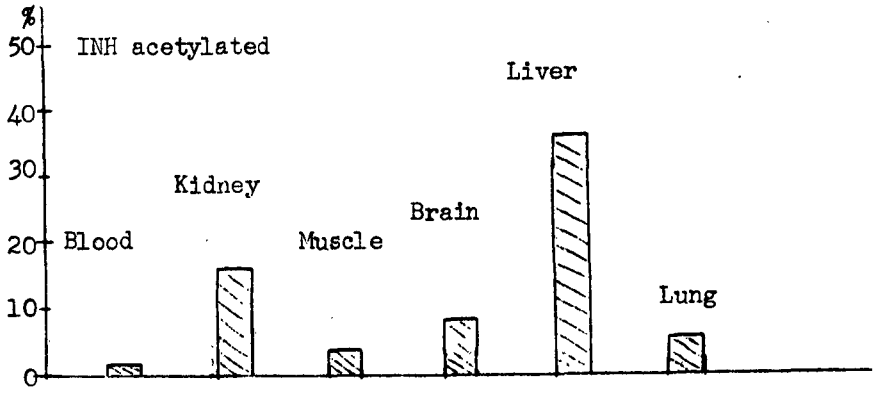


Fig. 31

Relationship between oxidation and acetylation of INH
Comparison among homogenates of various kinds of chicken organs

Organ	Oxidation (µl)	Acetylation (%)	Acetylation/oxidation
Blood	14	2.9	0.207
Kidney	164	16.5	0.100
Muscle	34	3.8	0.112
Brain	72	8.3	0.116
Liver	171	37.4	0.213
Lung	33	5.5	0.167

Average of 5 animals

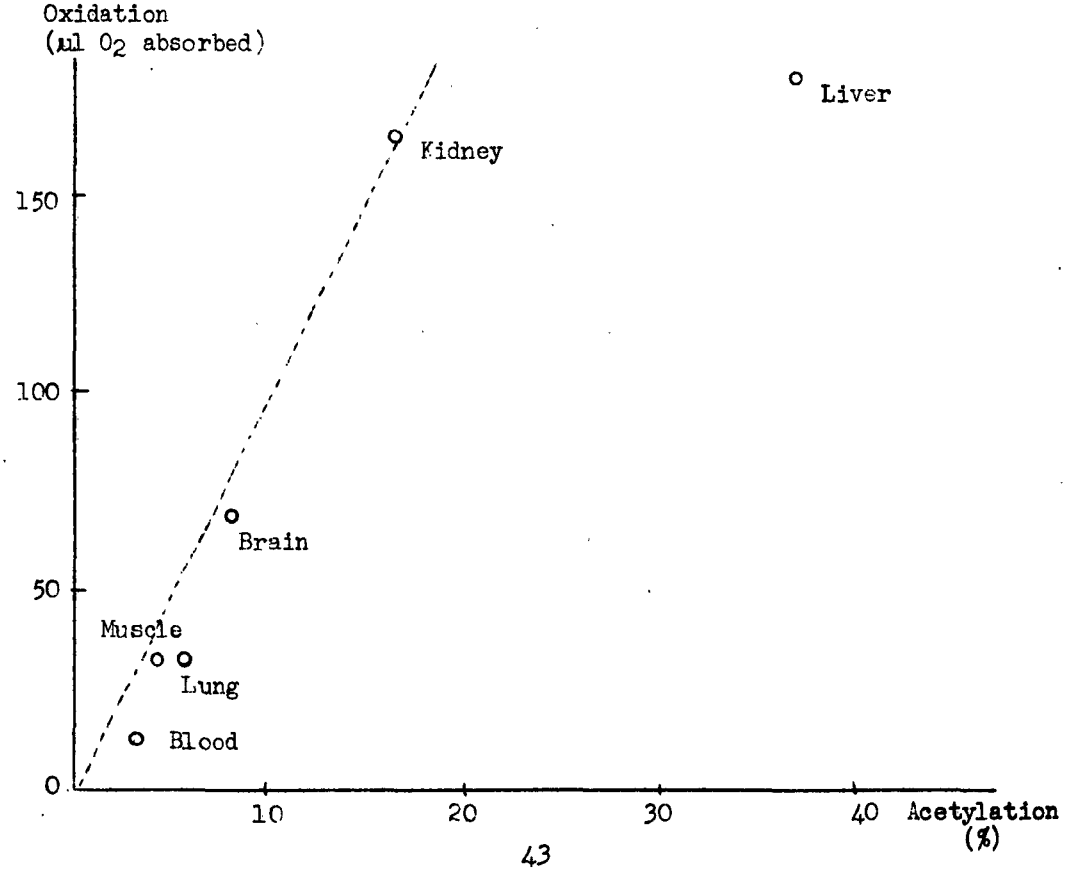


Fig. 32 Sulfisoxazole acetylating activity of liver homogenate
Comparison of various species of animals

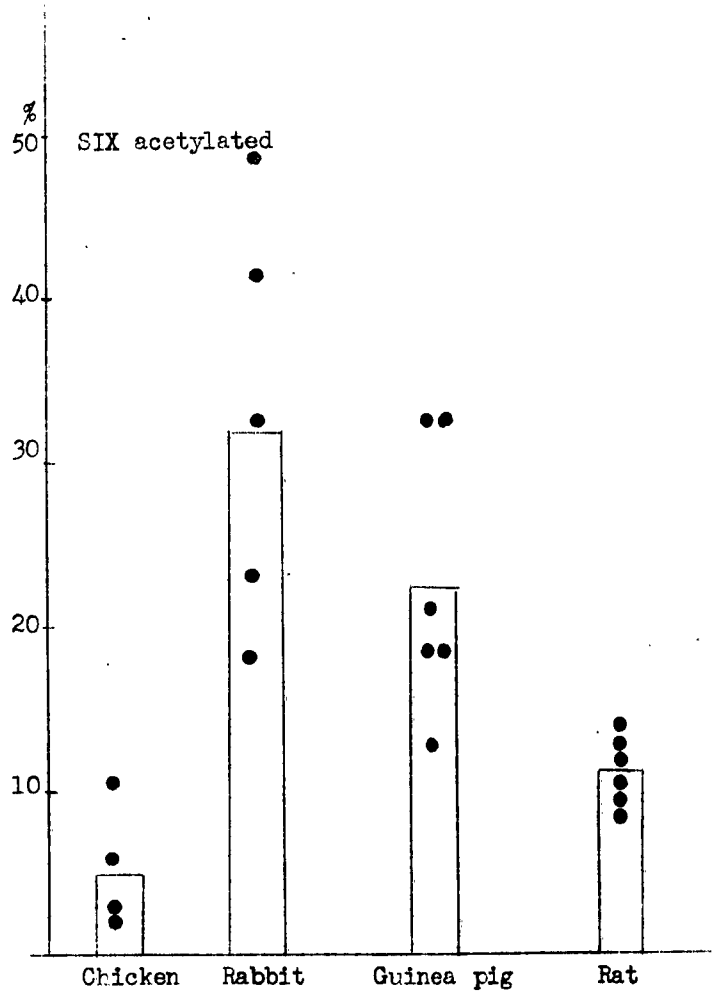


Fig. 33 PABA acetylating activity of liver homogenate

Comparison of various species of animals

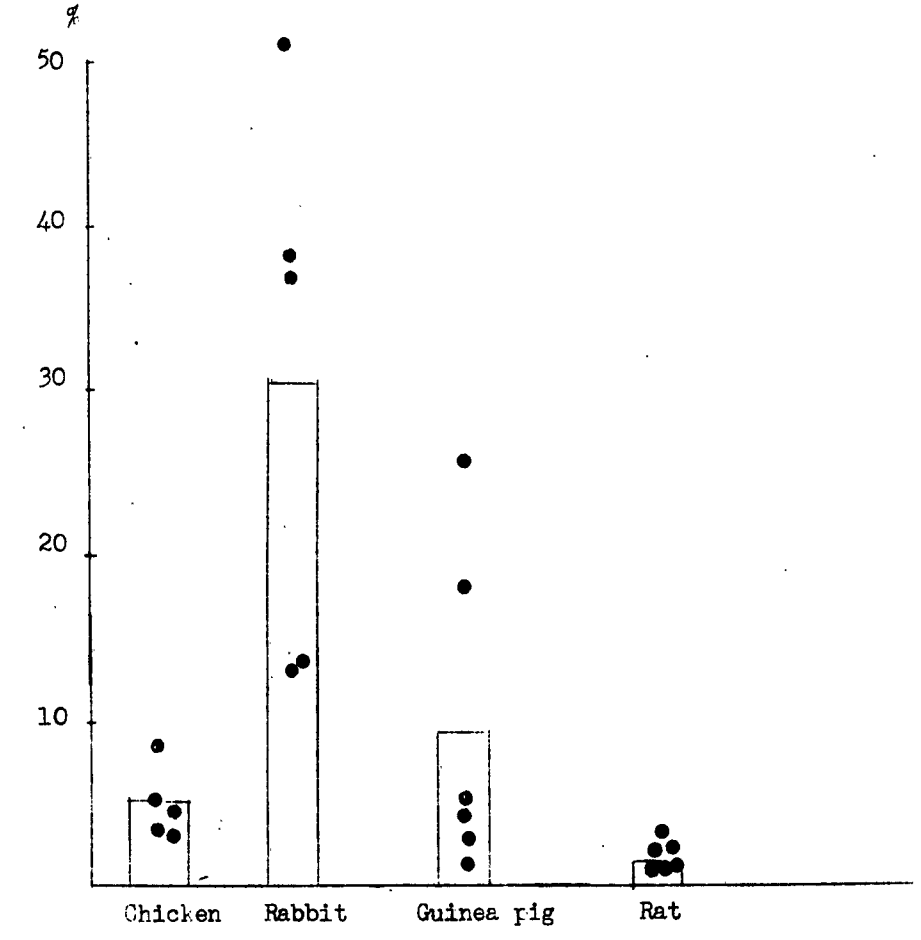


Fig. 34 Acetylation of INH, Sulfisoxazole (SIX) and PABA by pigeon liver homogenate

Exp.	INH	SIX	PABA
No.1	34.3%	-	-
2	57.5	2.7	-
3	55.5	0.5	69.0
4	80.2	13.3	80.2
5	41.8	0.0	48.2
6	26.6	-	-
7	73.0	7.0	79.0
Aver.	52.7	4.7	69.1

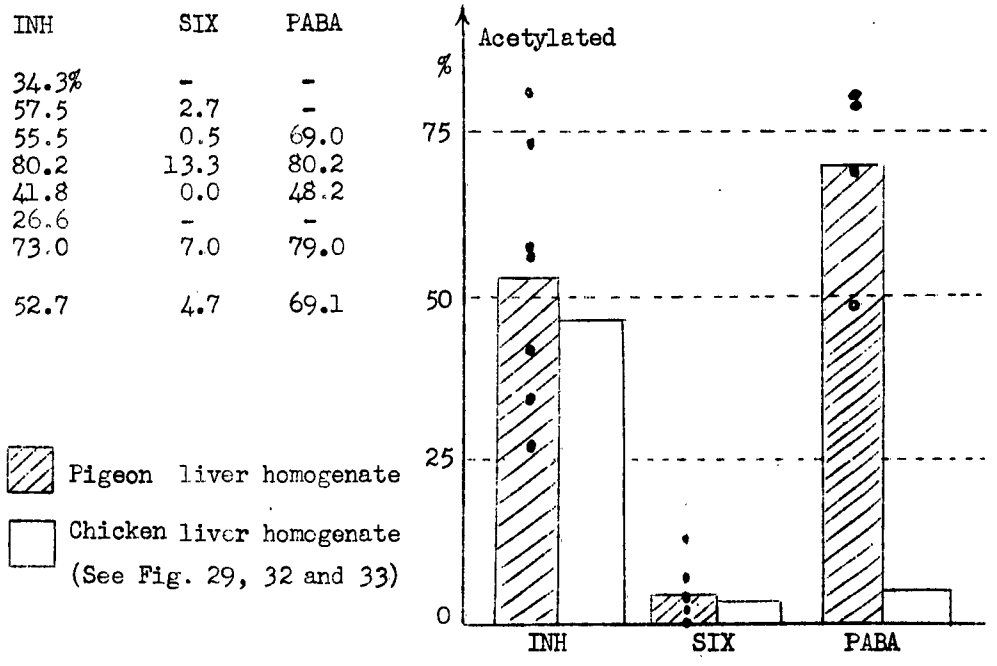


Fig. 35 Comparative study on acetylation of sulfanilamide (S.A.), sulfisoxazole (SIX) and INH by pigeon liver homogenate

	S.A.	SIX	INH
Exp. No. 1	43.1%	1.5%	32.8%
2	32.7	1.0	40.4
Average	37.9	1.25	36.6

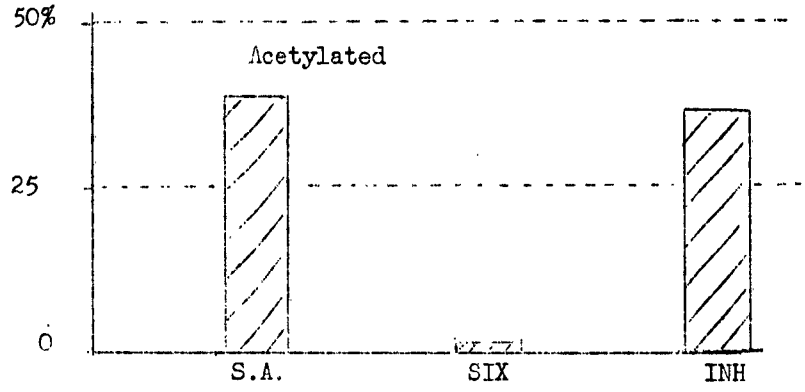


Fig. 36 Comparison between acetylation rates of sulfanilamide (S.A.) and INH by chicken liver homogenate

	S.A.	INH
Exp. No. 1	12.5%	26.0%
2	11.7	28.0
3	16.1	25.7
4	12.9	-
Average	13.3	26.6

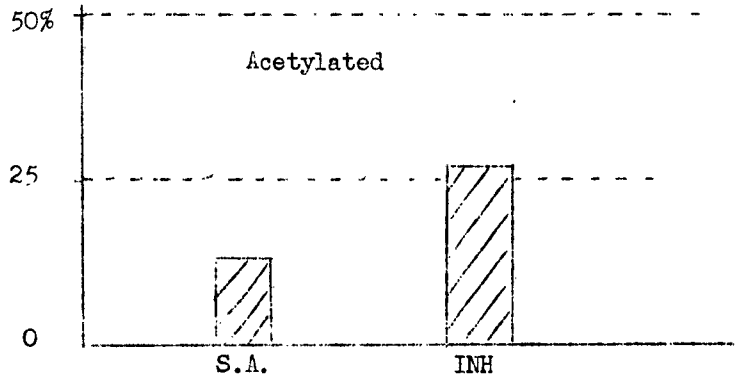


Fig. 37 Relationship between acetylation of INH and oxidation in liver homogenate of several kinds of animals

Animal species	No. of experiments	O_2 Uptake	INH Acetylation	Acetyl/O_2
Pigeon	7	193 μl	52.7%	0.27
Chicken	5	335	46.7	0.14
Rabbit	5	146	22.2	0.15
Guinea pig	5	250	5.7	0.02
Rat	5	306	0.2	0.00

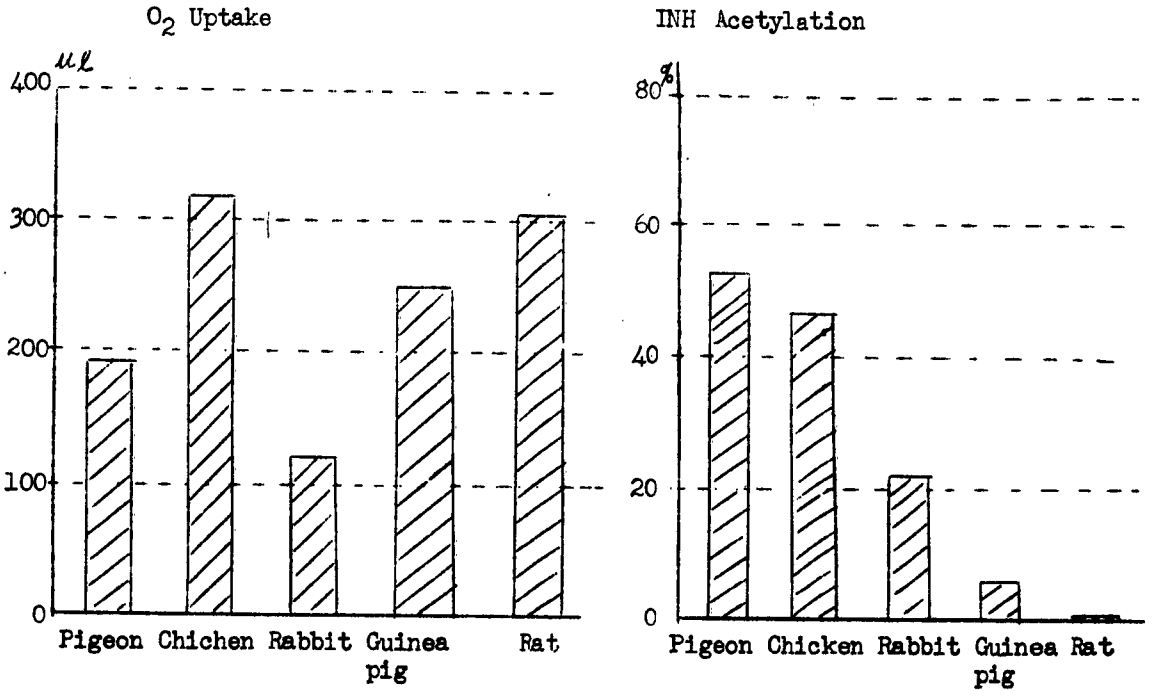


Fig. 38

Relationship between oxidation and INH acetylation in liver homogenates of several animal species

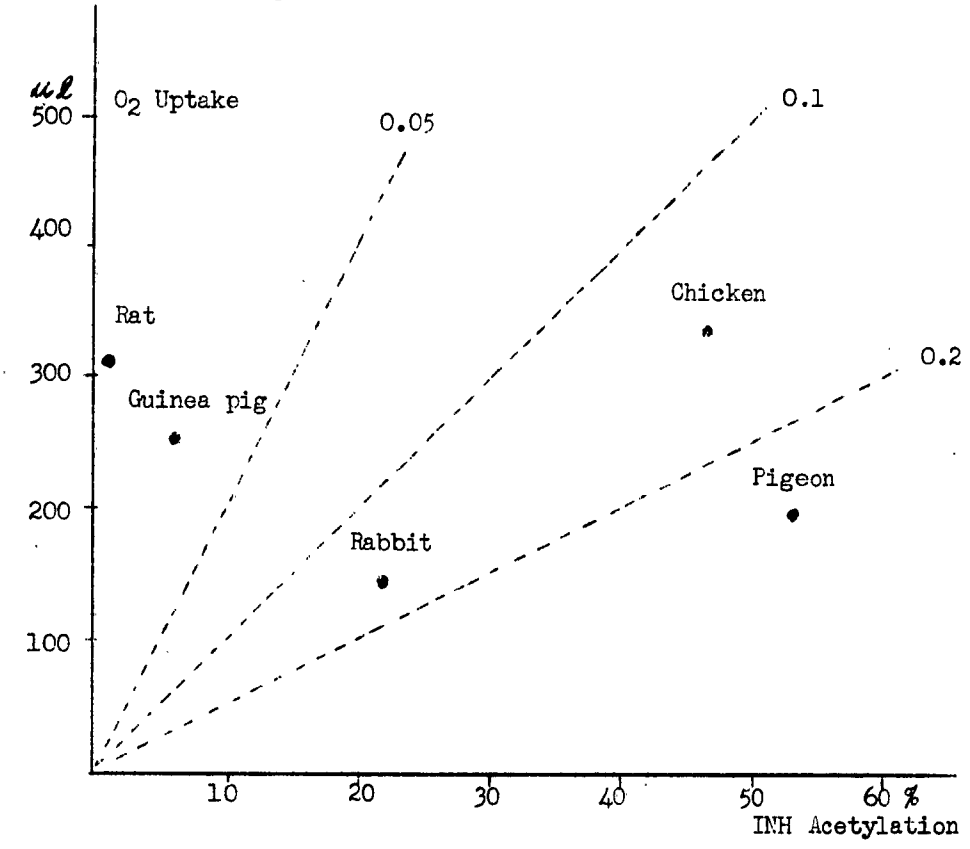
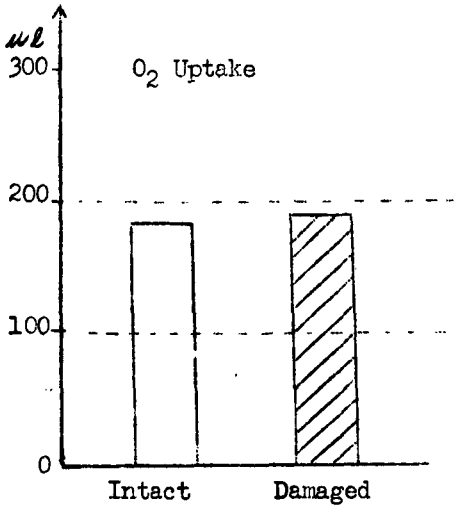


Fig. 39 Relationship between oxidation and acetylating activity in the case of experimentally induced liver damage in chicken

	O_2 Uptake		Acetylation	
	Intact	Damaged	Intact	Damaged
Exp. No.1	171.0 µl	119.0 µl	25.6%	9.2%
2	176.5	268.5	28.0	4.2
3	200.5	182.0	25.7	9.5
Average	182.7	189.8	26.5	7.6



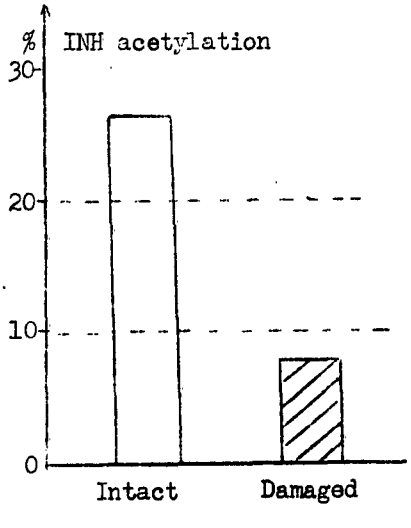
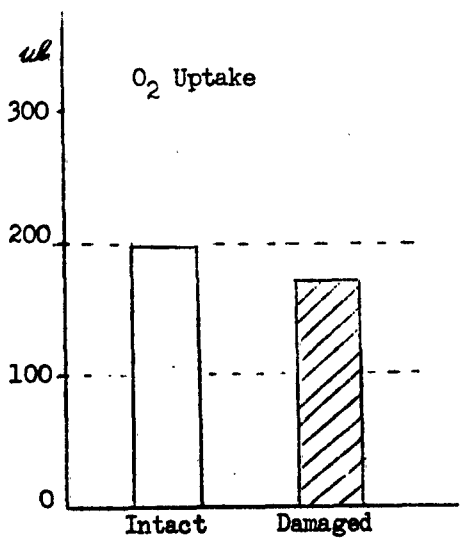


Fig. 40 Relationship between acetylation of sulfanilamide and oxidation in experimentally damaged liver of chicken

	O_2 Uptake		Acetylation	
	Intact	Damaged	Intact	Damaged
Exp. No.1	158.5 ul	103.0 ul	12.5%	0.5%
2	202.0	232.0	11.7	0.3
3	238.5	172.5	16.1	7.2
4	196.0	-	12.9	-
Average	198.7	169.2	13.3	2.7



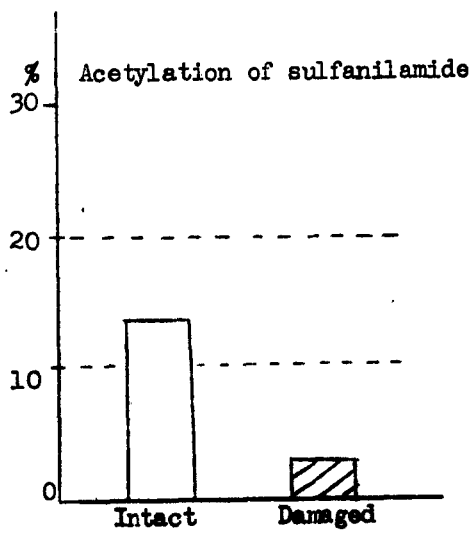


Fig. 41 Intracellular localization of INH acetylating principle

Experiment number	Fraction			
	Nucleus	Mitochondria	Microsome	Supernatant
1	2.7	2.6	14.0	23.4
2	5.1	0.7	27.3	39.0
Average	3.9	1.7	20.7	31.2

(Figure indicates γ INH acetylated /mg N)

Fig. 9

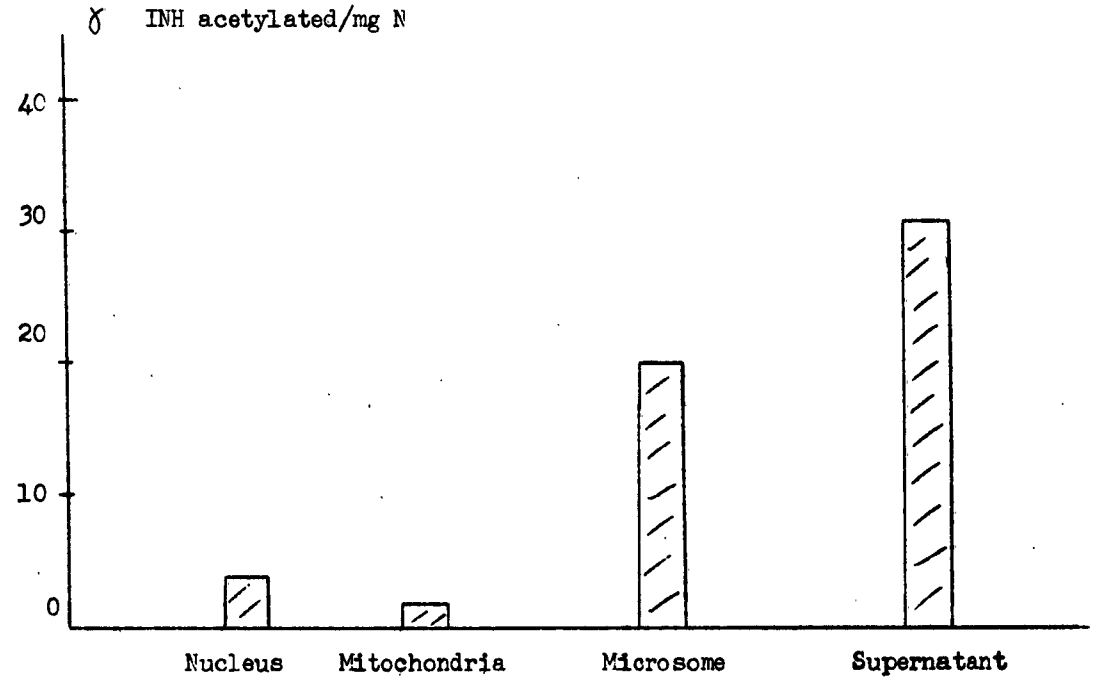


Fig. 42 Diagram of carbohydrate metabolism

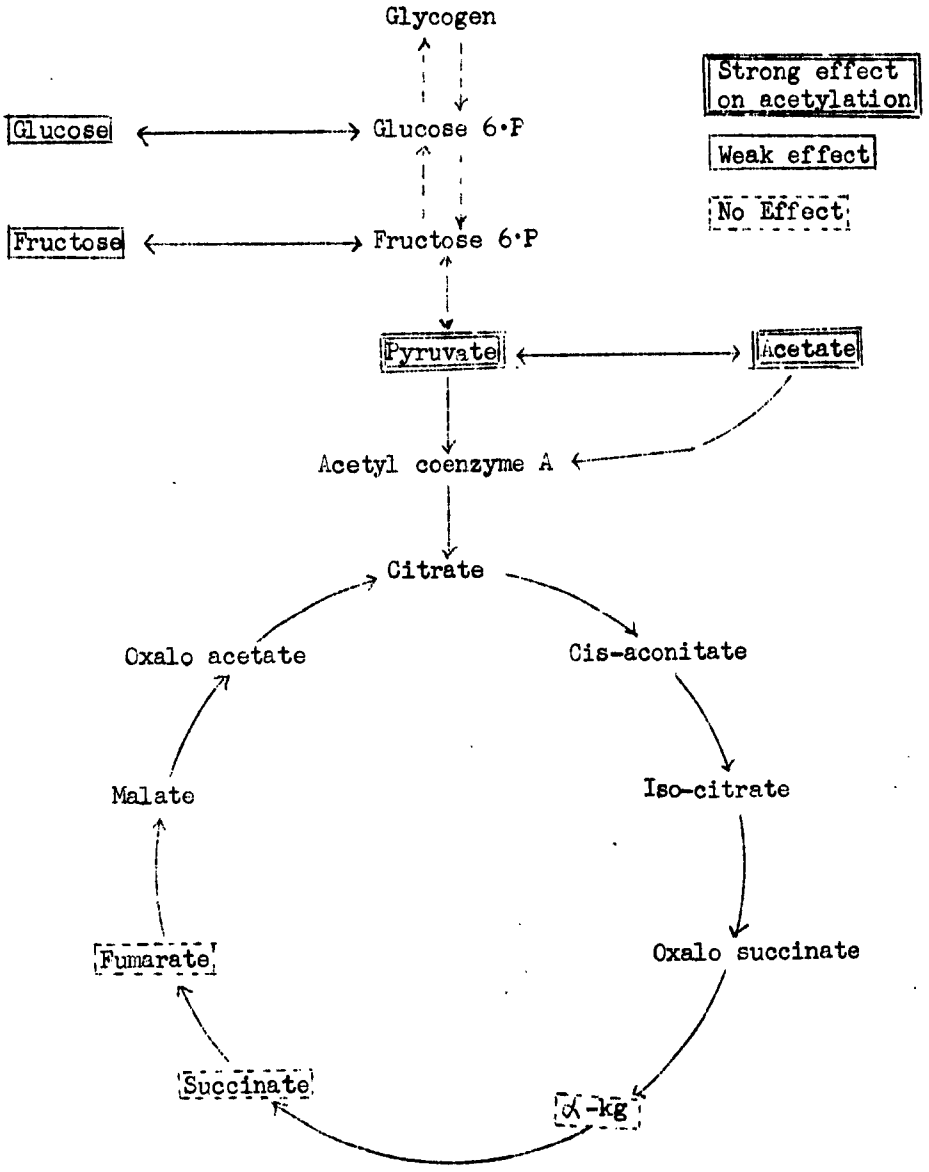


Fig. 43 Effect of increased dose of acetate on acetylation of INH by chicken liver homogenate (average of 2 experiments)

Acetate	Acetyl INH formed	Acetate effect	%
(-)	29.1 %	-	-
0.02 mM	67.7	38.6 %	19.3
0.04 mM	81.9	52.8	26.4
0.08 mM	93.9	64.8	32.4

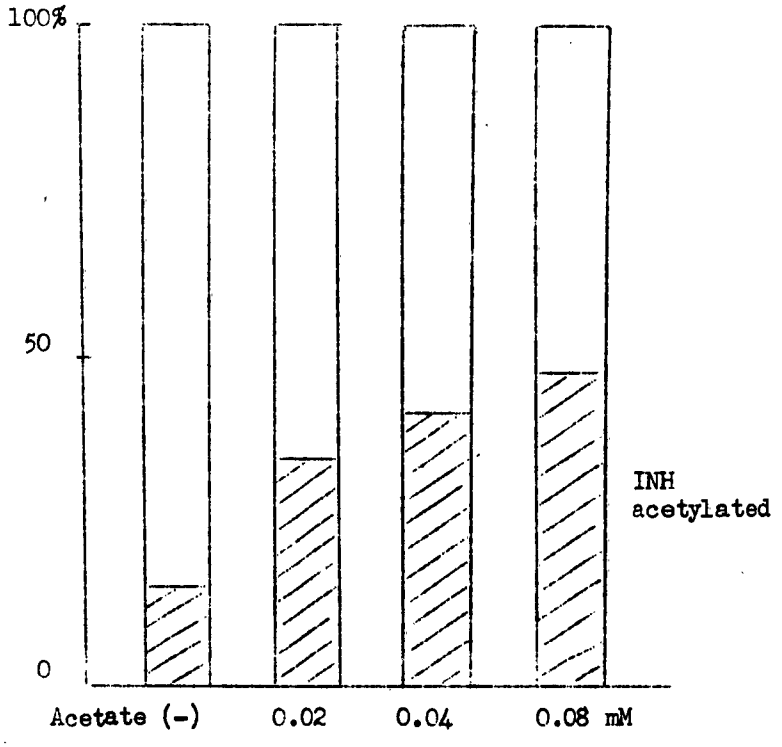


Fig. 44 Inactivation of INH by chicken liver homogenate

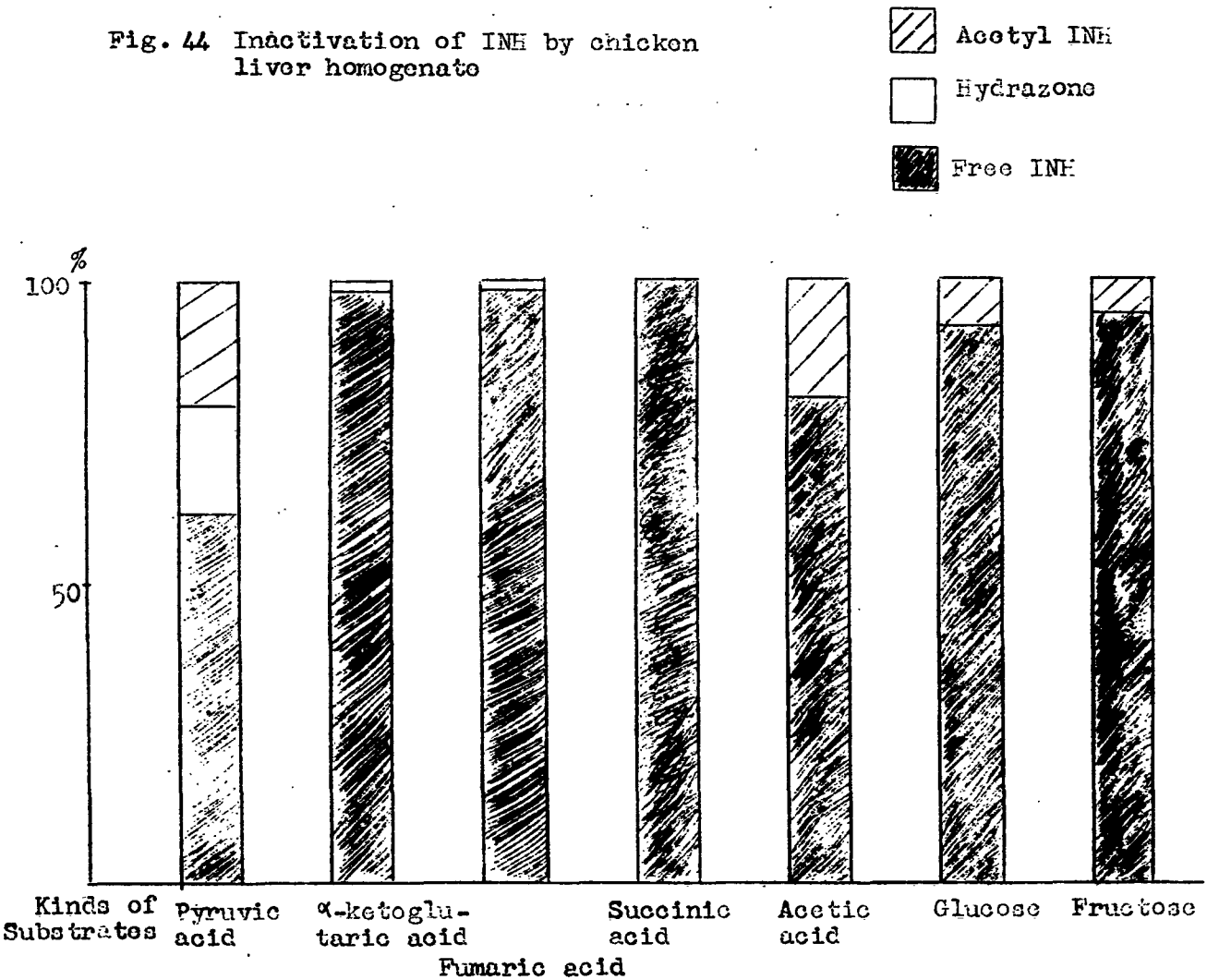
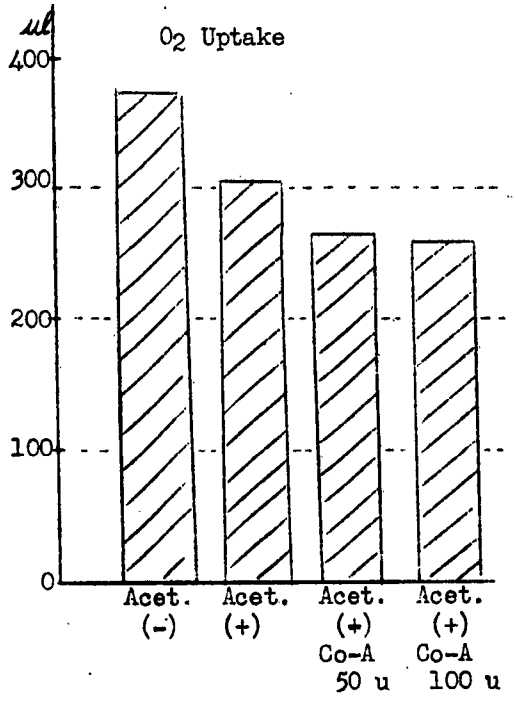


Fig. 45 Effect of addition of acetate or Co-A to rat liver homogenate on acetylation of INH

Dose of INH: 200 mg
Concentration of homogenate: 400 mg/ml

Acetate	Co-A	O_2 Uptake	Acetylation
-	-	373.0 μl	0.5%
0.04 mM	-	307.0	1.0
0.04	50 u	265.0	7.8
0.04	100	261.0	11.7



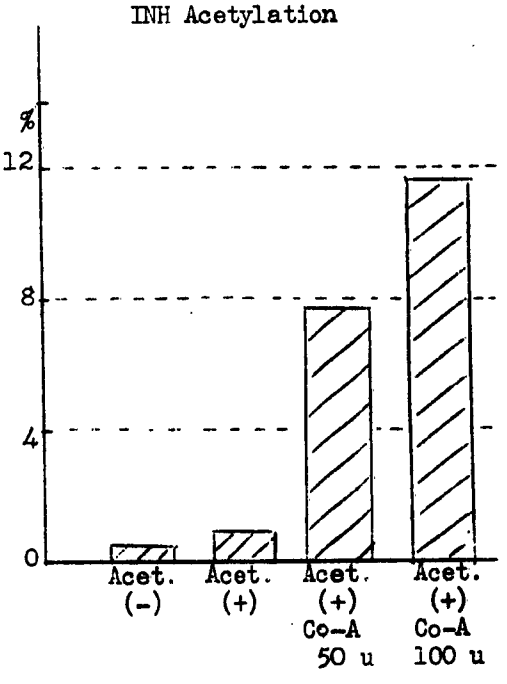
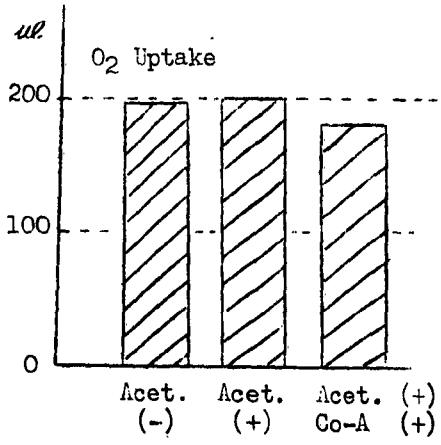


Fig. 46 Effect of addition of acetate or Co-A upon INH acetylation in pigeon liver

Dose of INH: 400 mg
Concentration of homogenate: 200 mg/ml

Mean of three experimental results

Acetate	Co-A	O_2 Uptake	INH Acetylation
-	-	197.8 ul	1.6%
0.04 mM	-	200.0	22.8
0.04	50 u	180.5	55.4



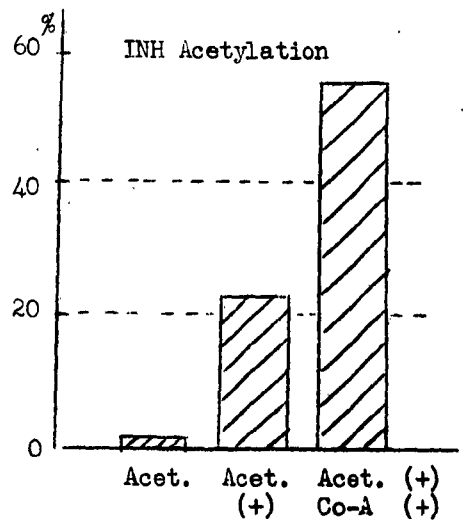


Fig. 47 Concentration-activity curves for Co enzyme A

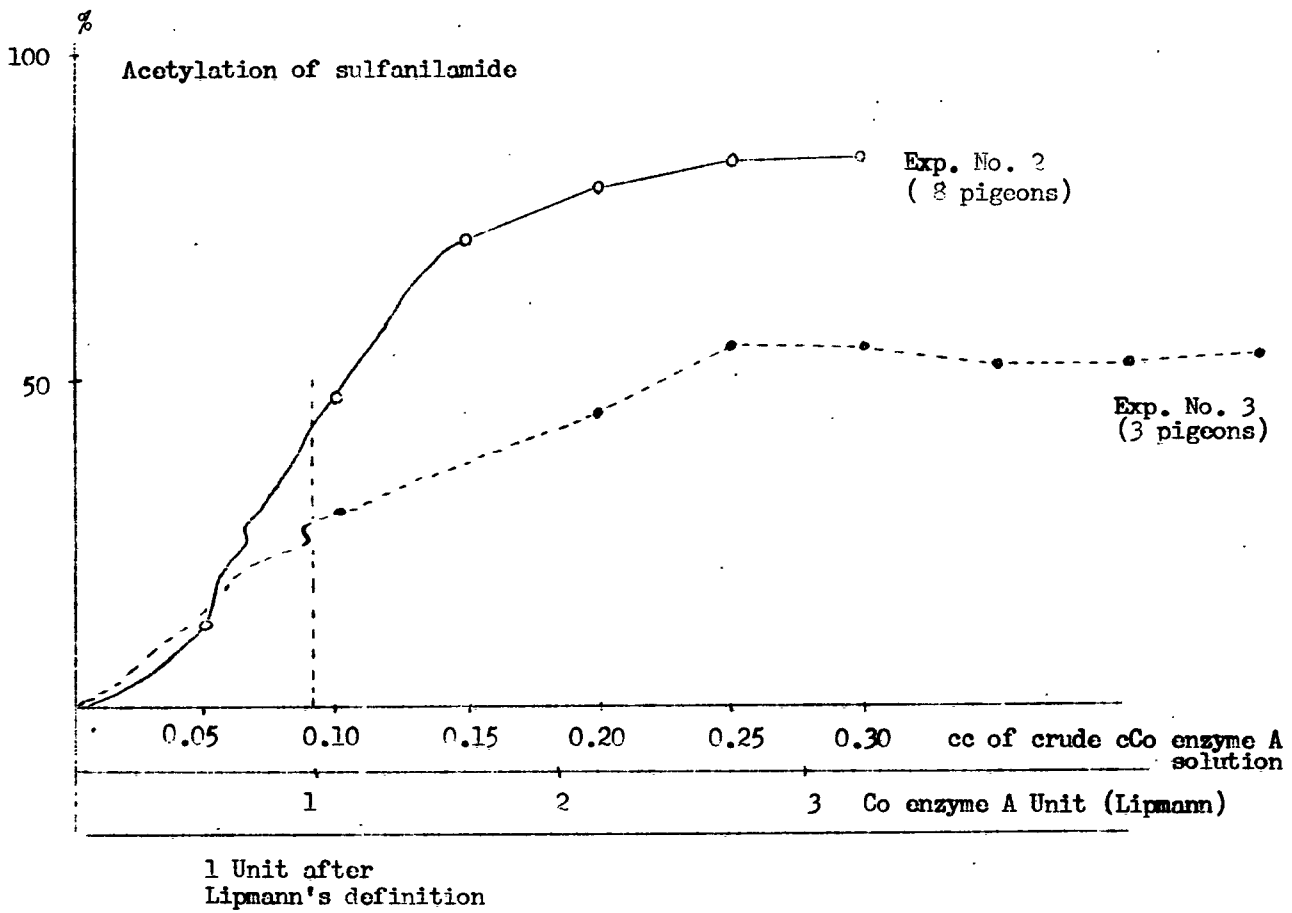
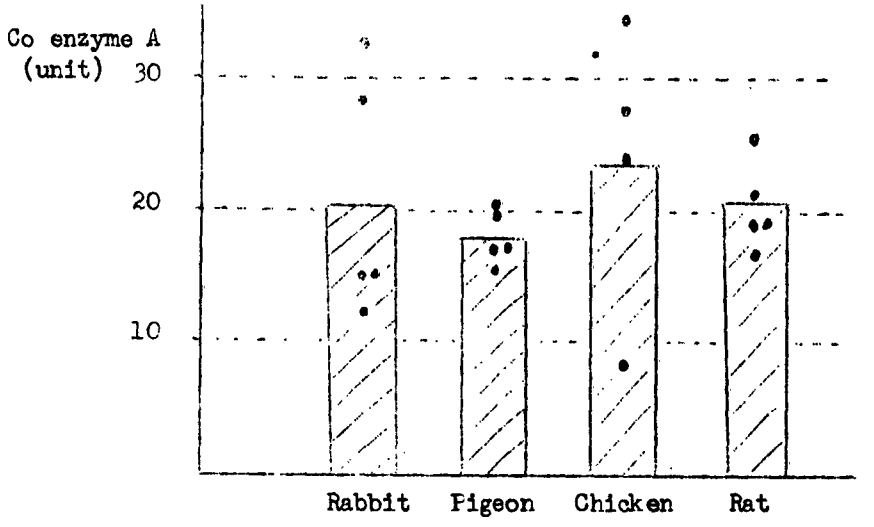


Fig. 48

Co enzyme A content of the liver

	Rabbit	Pigeon	Chicken	Rat
Exp. No. 1	32.9	20.3	27.7	21.0
2	28.1	15.3	7.7	16.7
3	15.0	19.5	34.6	25.4
4	12.8	17.0	23.5	18.9
5	14.0	17.3	-	18.5
Average	20.6	17.9	23.4	20.1

(unit)



IV. DISCUSSION

1. RACIAL DIFFERENCE IN ISONIAZID INACTIVATION

Our genetical hypothesis on isoniazid inactivation is based upon the observation of natural segregation of the three genetical traits (rapid, intermediate and slow inactivation). The segregation is easily and distinctly demonstrated at least in the case of Japanese when we take 6 hour plasma level of the biologically active isoniazid determined by the vertical diffusion method after ingestion of 4 mg per kg body weight of isoniazid. In other words, the trimodal frequency distribution curve is definitely established for Japanese, and the shapes of the two curves drawn up on the basis of the values for two different groups of Japanese are almost the same. On the contrary, Knight et al (7) and Evans et al (8) measured the concentration of isoniazid in blood by means of a bioassay method which dealt with serial dilution of serum or a kind of biochemical method and observed bimodality of the distribution curve. It is the reason why they do not agree fully with our genetical hypothesis.

In the present research, we were successful in establishing trimodality of the frequency distribution curves as to Chinese in Formosa and Thai people in which the incidences of "Slow" characters were much higher than in Japanese. The observation with regard to Thai people is all the more important because the percentage of the alleles controlling isoniazid inactivation in this race is very close to that of the Whites. It is also worthy of notice that two values for gene frequency in Thai determined on two different occasions by us coincided with each other fairly well.

Adding to that, we observed trimodality also in the children of mixed Japanese and Caucasian or negro parentage the majority of whom were "Intermediate" character.

Our hypothesis on "cline" with regard to frequency of the alleles of isoniazid inactivation was verified again by our present research which included Thai and Chinese. To investigate whether the same trend is also the case with inhabitants in other continents than the Far East is a problem for our future study.

2. THERAPEUTIC EFFECT OF THE ISONIAZID TREATMENT RELATED TO THE BIOLOGICALLY ACTIVE ISONIAZID CONCENTRATION

A number of investigators have reported already on the relationship between blood level of isoniazid and clinical efficacy of isoniazid treatment of pulmonary tuberculosis. But the only reports which are concerned with the controlled trials on the randamized subjects are those by the Veterans Administration Study (9) in the USA, the Chemotherapeutic Center of Madras (10) in India and Scotish Tuberculosis Society (11). But in these studies the blood levels were determined, to our regret, only after a fixed dose, for example 4 mg/kg body weight of isoniazid, regardless of the actual therapeutic dose and form of administration of this drug during treatment. It is quite reasonable for the purpose of the genetical study to classify the patterns of

isoniazid metabolism based on the plasma or serum concentration of isoniazid after the fixed dose, but it is more reasonable, I believe, to take the blood levels after the dose of the drug strictly identical to the actual dose which is administered every day to individual patient under study, if we want to correlate the blood levels with the clinical effect. For example, if 0.3 g of isoniazid and 12 g of PAS were prescribed daily to a patient and he took these two drugs simultaneously in three divided doses, we had better measure the biologically active isoniazid level after a single dose of 0.1 g of isoniazid and 4 g of PAS instead of 4 mg/kg of isoniazid alone.

We have conducted six series of the comparative clinical trials on chemotherapy of pulmonary tuberculosis since 1958, allocating five or six kinds of chemotherapeutic regimens at a time to the newly admitted patients without previous chemotherapy. In the present research we studied the correlation between clinical efficacy and blood level of isoniazid not only after the dose of 4 mg/kg body weight but also after the actually prescribed dose in a part of patients treated by isoniazid + PAS, isoniazid + PZA or isoniazid + sulfonamide.

We failed to establish any relationship between the rate of sputum conversion and the blood level, although we tried to express the latter in various ways. But in the case of isoniazid and PAS treatment in the second and third series, the regression of cavity was slightly related to the blood level, especially if we administered the actually prescribed dose to the subjects under examination but the correlation is not statistically significant.

We compared the ordinary dose of isoniazid with the very low dose but as the subjects admitted to the trial were too few, we could not establish the necessary and sufficient concentration of isoniazid in vivo below which no clinical efficacy was expected.

In conclusion as far as our own clinical research are concerned, a close relationship between clinical effect and blood level of isoniazid was not recognized and we could not have a solid foundation for high dose isoniazid regimen even in the case of Japanese the majority of whom were rapid inactivators.

3. BIOCHEMICAL STUDIES ON ACETYLATION IN ANIMAL TISSUE

It is beyond question that inactivation of isoniazid is a genetical character and it differs from person to person but constant in an individual. Racial and species difference in isoniazid metabolism are also observed. What kind of biochemical process do such differences depend on?

By means of Warburg's conventional method, we tried at first to compare acetylating activities of liver homogenates of different kinds of animals with each other and found that chicken liver acetylated isoniazid most actively and rat liver lacked the activity completely. Addition of acetate to the reaction system accelerated the acetylation in chicken and rabbit liver but it was of no effect on guinea pig and rat liver. In other words, the difference in acetylating activity between chicken and rat could not be explained by different amount of acetate in the liver of these two species.

Among various kinds of animals which we examined, chicken showed the highest activity as to isoniazid acetylation and among various kinds of chicken organs, the liver acetylated isoniazid most markedly. But we found that acetylation of sulfisoxazole and PABA by chicken liver was very weak. Although isoniazid inactivation rate decreased in the order of pigeon, chicken, rabbit, guinea pig and rat, acetylation of sulfisoxazole and PABA by chicken liver was the weakest or second weakest among these animals. On the other hand high acetylating activity of pigeon liver with regard to sulfanilamide and PABA had been reported by Lipmann and other investigators (5,6). We observed PABA was readily acetylated in pigeon liver but sulfisoxazole remained almost unacetylated also in the case of pigeon liver homogenate.

We compared acetylation rates of sulfisoxazole and sulfanilamide in chicken and pigeon liver homogenate and found that sulfanilamide was much more easily acetylated than sulfisoxazole both by chicken and pigeon liver homogenate. Therefore, we came to the conclusion that the low acetylating activity of chicken liver homogenate as to sulfisoxazole did not depend on the animal species but on the kind of drug, while weak activity of acetylation of PABA in chicken liver was due to the animal species.

Some investigators reported already that the liver was the sole or the most important organ with respect to acetylation of sulfonamide and PABA and our observation on isoniazid acetylation coincided, generally speaking, with these reports. Further more, some clinical investigators suspected or even believed that there was more or less close relationship between acetylation of isoniazid and other kinds of liver functions in the patients suffering from several kinds of liver diseases, although we were not successful in observing such a correlation. In the present research we found that in the chicken liver which was experimentally damaged by means of CCl_4 injection, no change in O_2 uptake was observed, while acetylation of isoniazid and sulfanilamide decreased remarkably and the rate of drop was almost same as to these two kinds of drugs.

We observed that addition of glucose, fructose, acetate or pyruvate caused more or less definite rise in the rate of acetylation of isoniazid by chicken liver homogenate, while the substances which were members of TCA cycle such as α-ketoglutaric acid, fumaric acid and succinic acid produced no effect on acetylation. In the case of guinea pig and rat liver which showed only negligible activity initially, even when acetate was added to the reaction system, no increase in acetylation was established, although remarkable rise was observed in chicken liver which originally acetylated isoniazid and sulfonamide very rapidly. Even if we put Co enzyme A into the reaction system in addition to acetate, rat liver acetylated isoniazid only to a slight degree and Co enzyme A content of liver was almost same, regardless of different grade of acetylating activity of different species of animals. Taking all these considerations into account, the tentative conclusion was reached that the species difference in isoniazid metabolism did not depend on amount of substrates and Co enzyme A but in the main on acetylase content of the tissue.

Our investigation from now on will concern with the cause of individual difference in isoniazid inactivation.

V. CONCLUSION

1. Trimodality of frequency distribution curves of biologically active isoniazid levels were established not only in Japanese but also Chinese in Formosa, Thai people and half-bloods between Japanese and Caucasians or negroes.

2. Cline from north to south as to frequency of the alleles controlling isoniazid inactivation was established with regard to several Asiatic races.

3. Correlation between clinical effect of isoniazid treatment of pulmonary tuberculosis and isoniazid concentration in blood plasma after a fixed dose of test drug and also after a single therapeutic dose was investigated, and it was revealed that there was not a statistically significant difference between high and low dose regimen of isoniazid and also between cases with high and low blood levels of isoniazid.

4. Rate of isoniazid inactivation varies from animal species to species and decreases in the order of pigeon, chicken, rabbit, guinea pig and rat.

5. Acetylation of sulfisoxazole and PABA by chicken liver was the weakest or second weakest among these animal species contrary to expectation.

6. Pigeon liver acetylates PABA actively but sulfisoxazole only to the least extent, while sulfanilamide is inactivated in chicken and pigeon liver quite markedly.

7. The liver is the most important tissue as to acetylation and acetylating enzyme is located in the main in supernatant fraction of cell. Remarkable drop in acetylation is observed after the artificially induced liver damage.

8. Substances which are the member of TCA cycle have no effect on acetylation while glucose, fructose and especially substances which is located close to acetyl Co enzyme A in the pathway of carbohydrate metabolism such as acetate and pyruvate accelerate acetylation of isoniazid remarkably.

9. In the case of guinea pig and rat liver homogenates which have only negligible activity of acetylation initially, addition of acetate or Co enzyme A causes only slight increase in the activity.

10. Co enzyme A content of the liver is almost same in different species of animals regardless of difference in acetylating activity

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